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EFFECTS OF REDUCING AGENTS AND UREA ON
THE CONSTITUENTS OF GLUTEN

by

[illegible] YANG

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR
THE DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF FOOD SCIENCE

EDMONTON, ALBERTA

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THE UNIVERSITY OF ALBERTA

EFFECTS OF REDUCING AGENTS AND ENZYMES ON THE CONSTITUENTS OF GLUTEN

by

HUNG-JU YANG

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES
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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Effects of reducing agents and enzymes on the constituents of gluten" submitted by Hung-Ju Yang in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

ABSTRACT

Fractional precipitation was used in a study of the constituents of gluten, of bisulfite-treated gluten, and of sulfite-treated gluten, dispersed in sodium salicylate.

The results obtained with fractional precipitation indicate that the disulfide bonds of the gluten molecules were ruptured on prolonged treatment with sulfite, but only slightly, if at all, with bisulfite. Bisulfite treatment apparently resulted only in disaggregation of gluten proteins.

The amino acid content of each fraction was determined. The results indicate that fractions differ progressively in the distribution of amino acids regardless of the treatment to which gluten was subjected. Comparisons of the corresponding fractions confirm the idea that intermolecular disulfide bonding is responsible for the high molecular weight of glutenin, but no evidence was obtained to support the idea that glutenin is built up from gliadin residues.

The effects of papain and pepsin on whole gluten and on the fractions were also studied. The results indicate that gluten was digested extensively by pepsin or papain, but these two enzymes act differently. Precipitates formed in the digests of all fractions of untreated gluten but in

only the more insoluble fractions of gluten treated with bisulfite. The amino acid distribution of the precipitates was quite different from that in the original fractions, but also differed progressively in successive fractions.

Studies of the peptides in the enzymic digests of the fractions indicate that there was essential similarity in the chromatographic patterns of papain digests of all the more soluble fractions, but there were differences in the patterns for the more insoluble fractions.

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TABLE OF CONTENTS

Page

INTRODUCTION.. 1

LITERATURE REVIEW

The constituents of gluten................................ 3

The amino acid composition of gluten and its components..........10

The effect of reducing agents on gluten proteins........ 12

The effect of proteolytic enzymes on gluten proteins.... 14

MATERIALS AND METHODS

Preparation of gluten and gluten dispersions........... 19

Fractionation of dispersed gluten....................... 20

Determination of amino acids............................ 22

Enzymic digestion of gluten............................. 23

Determination of non-protein nitrogen, terminal amino group nitrogen, and free amino acid nitrogen......... 24

Ion exchange chromatography of digests.................. 24

Determination of ribonucleic acid....................... 27

RESULTS

Fractionation of dispersed gluten from 10% sodium salicylate solution with or without added bisulfite or sulfite...................................... 30

Fractionation of dispersed gluten.................... 30

Analysis of the fractions............................ 33

TABLE OF CONTENTS (Cont'd.)

Page

Fractionation of dispersed gluten from aluminum lactate solution.................................... 44

Effect of proteolytic enzymes on gluten and its fractions...46

Enzymatic hydrolysis of dispersed gluten...............46

Enzymatic hydrolysis of gluten fractions...............55

Polypeptide analysis of enzymic digests of gluten fractions...63

DISCUSSION...70

REFERENCES...81

LIST OF TABLES

TABLE Page

I Composition of buffers used for peptide analysis..... 25

II Buffer composition of autograd chambers............... 26

III The amino acid composition of gluten and its fractions obtained from gluten dispersed in 10% sodium salicylate without added bisulfite............ 35

IV The amino acid composition of gluten dispersed in 10% sodium salicylate solution with added bisulfite, and of its fractions obtained by the addition of magnesium sulfate.................................. 38

V The amino acid composition of the fractions obtained from gluten dispersed in 10% sodium salicylate solution after prolonged treatment with sulfite.............................. 42

VI The amino acid composition of the fractions obtained from aluminum lactate solution by varying the pH.... 45

VII Digestion of dispersed gluten with pepsin, papain, and trypsin................................ 48

VIII Free amino acids liberated from Fraction D by the action of pepsin............................ 53

IX The amino acid composition of the precipitates from Fractions I to VII........................... 57

LIST OF TABLES (Cont'd.)

TABLE Page

X The amino acid composition of the precipitates from Fractions IIB, IIIB, A, B, and C.............. 58

XI Amino acid content of total protein in fractions and of the corresponding precipitates.............. 61

XII The nucleic acid content of the precipitates obtained from peptic digests of Fractions A, B, and C... 62

XIII The peaks in Figure 9 identified to be free amino acids.. 68

XIV Recent amino acid analyses of gluten components..... 74

LIST OF FIGURES

FIGURE Page

1. Precipitation of dispersed gluten with $MgSO_4$...... 32
2. Amino acid content of gluten fractions obtained from gluten dispersed in 10% sodium salicylate without added bisulfite............... 36
3. Amino acid content of gluten fractions obtained from gluten dispersed in 10% sodium salicylate after prolonged treatment with bisulfite.......... 39
4. Amino acid content of gluten fractions obtained from gluten dispersed in 10% sodium salicylate after prolonged treatment with sulfite........... 43
5. Non-protein nitrogen increase in gluten during enzymatic hydrolysis....................... 49
6. Terminal amino nitrogen appearing during enzymatic hydrolysis............................ 50
7. The chromatographic patterns obtained for enzymic digests of gluten fractions using Aminex 50W-X2.............................. 65
8. The chromatographic patterns obtained for enzymic digests of gluten fractions using Beckman type AA-15......................... 66
9. The chromatographic patterns obtained for peptic digest of Fraction D...................... 69

INTRODUCTION

Wheat gluten is exceedingly complex in constitution. The earliest workers accepted Osborne's suggestion that gluten consisted of two proteins, gliadin and glutenin. For the last three decades, some investigators have believed that gluten consisted of a series of proteins differing progressively in solubility and other properties, while others favored the view that gluten consisted of a limited number of components which interacted in some manner to form a complex whose parts differed slightly but progressively in properties.

With the recent development of new research methods and instruments, the constitution of gluten has begun to be better understood. Progress in various fields has been successively reported, and most of the results have been based on the properties of gluten in aqueous acid media. Electrophoretic techniques and ion exchange chromatography applied to the study of acid dispersions of gluten have clearly demonstrated the heterogeneity and molecular discreteness of gluten components, particularly those making up classical gliadin. Moreover, the concepts that glutenin is built up from gliadin residues and that intermolecular bonding is responsible for its very high molecular weight resulted from studies of gluten dispersed in acid media.

Consequently, it seemed desirable to check these results with gluten dispersed in a neutral solvent, in which gluten is more stable and in which less interaction of the proteins appears to occur. The present study was undertaken to obtain

such information by: (a) an examination of the amino acid composition of the fractions obtained from gluten dispersed in sodium salicylate, (b) a study of the changes in the behavior of the dispersion when reducing agents are added, and (c) a study of the response of gluten and its fractions to enzymic action. For comparative purposes, some experimental work was carried out on gluten dispersed in aluminum lactate, the acid medium most commonly used by other workers in recent studies.

LITERATURE REVIEW

The constituents of gluten.

Extensive and systematic studies on wheat protein and its fractions were begun by Osborne and his associates in the early 1900's. The results of these comprehensive studies were summarized in his monograph "The Proteins of the Wheat Kernel" (1) published in 1907. His essential conclusions were: (a) that the proteins of flour can be separated into five fractions based on solubility; gliadin, soluble in 60-70% alcohol, glutenin, soluble in dilute acid and dilute alkali, a neutral salt-soluble edestin (globulin), a water-soluble leucosin (albumin), and an ill-defined proteose; and (b) that the actual gluten protein is an intimate mixture of two well-defined proteins, gliadin and glutenin, present in nearly equal amounts.

Subsequent studies (2-4) attempted to measure the content of glutenin in gluten based upon its extraction with dilute alkali, following the preliminary removal of the non-gluten proteins and gliadin by salt solution and 70% alcohol, respectively. Results obtained showed wide inconsistencies.

Csonka and Jones (5) reported obtaining two components from the glutenin fraction by salting out the glutenin from alkaline extracts with ammonium sulfate rather than precipitating by neutralization of the alkali. They designated these as alpha and beta glutelins.

Blish and Sandstedt (6) reported that the composition of the glutenin fraction varied in accordance with the concentration of alkali used for dispersion, and also showed that

glutenin, precipitated from dilute acetic acid dispersion by the addition of alcohol, was different in chemical composition from that prepared using alkali.

Haugaard and Johnson (7) studied the gliadin fraction, and reported that gliadin can be fractionated from 60% alcohol dispersion by varying the temperature. Three fractions were obtained at the temperatures of 0°C and -11°C, and these fractions showed significant differences in viscosity and optical rotation, but no appreciable differences in amino acid composition. They concluded that gliadin is inhomogeneous.

Cook and Alsberg (8) found that gluten can be dispersed in 30% urea and the glutenin fraction is precipitated by adding salt or by dilution. Cook (9) studied the fractionation of gluten dispersions by fractional precipitation at salt concentrations varying from 0.1 to 0.5 saturation of magnesium sulfate. Six fractions varying continuously in their gliadin and glutenin content were obtained. It was suggested that there is no sharp dividing point between gliadin and glutenin fractions. Later, Sandstedt and Blish (10) fractionated gluten from dilute acetic acid dispersion by the addition of salt, with or without the addition of alcohol, or by neutralization of the acid. Fractions of varying composition were obtained, and according to these authors, sharp and definite boundaries of fractions were hard to get and certain trends of properties and behavior indicated that these fractions may be conveniently classified into three main groups, namely the glutenin group, the gliadin group, and an intermediate group, which they termed "mesonin" since it had intermediate properties.

McCalla and Rose (11), using 10% sodium salicylate solution as the dispersing agent, carried out a fractional precipitation of gluten by successive addition of magnesium sulfate. When the amount of gluten precipitated was plotted against the concentration of magnesium sulfate, a smooth curve was obtained, suggesting that gluten did not consist of distinct fractions such as gliadin, glutenin, and mesonin. Moreover, when the fractions were analyzed for amide-N and arginine-N content, they showed progressive and systematic variations in chemical composition. It was concluded that gluten, except for the most soluble fraction, is a simple protein complex which can be separated into a great many fractions differing systematically in both physical and chemical properties. This conclusion was further supported by the work of Spencer and McCalla (12) in which fractional solubility of gluten in sodium salicylate was employed. Further confirmation was obtained by McCalla and Gralén (13) on the investigation of molecular characterization of gluten and its fractions, using sedimentation and diffusion methods. It was shown that only part of the gluten in salicylate solution existed in molecularly dispersed form, and the remainder was present in various stages of aggregation.

Harris and his associates (14-18) have studied a number of gluten fractions and their relationship to baking strength. Various fractionation techniques such as thermal fractionation (14), fractional precipitation (15) according to the method of McCalla and Rose (11), and fractional precipitation by varying pH (17) have

been employed. None of these studies have given any results explaining the fundamental characteristics of the fractions. Stauffer, Banasik, and Harris (18) have reported the results of the amino acid distribution in the fractions obtained by adjusting the pH levels of lactic acid dispersion of gluten. Significant variations in the content of certain amino acids were found, but no conclusions were drawn from the results.

The study of proteins from wheat, barley, rye, and oats by Cunningham, Geddes, and Anderson (19) included the fractionation of gluten proteins, in which formic acid extracts of the flour were fractionally precipitated by the addition of a variety of salts of different pH levels. Their studies indicated that salt-protein complexes may be partially responsible for the known insolubility of the proteins and that the sigmoidal shape of the precipitation curves suggested that the constitution of gluten appears to be a complex of closely related proteins rather than a mixture of homogeneous fractions.

The use of electrophoretic techniques for the study of gluten as reported by Mills (20), indicated that four asymmetric peaks were obtained from gliadin in acetic-hydrochloric acid buffer. A more successful study was carried out by Jones, Taylor, and Senti (21) who obtained excellent symmetry of electrophoretic patterns for gluten by using aluminum lactate-lactic acid buffer. It was reported that gluten is composed of at least four major components and one minor component. A partial separation of the electrophoretic components was obtained by fractional precipitation of aluminum lactacte dispersions of gluten by progressively increasing the pH to

5.1, 5.2, 5.3, 5.4, and then by increasing ionic strength to 0.09.

The use of chromatographic techniques for the fractionation of gluten was described by Woychik, Dimler, and Senti (22), using a carboxymethylcellulose column and stepwise decreases in the pH of the eluting buffer from 3.4 to 1.5. Four fractions were obtained from aluminum lactate dispersions of gluten, and each fraction was further examined by electrophoresis. It was found that the fractions corresponded to the components obtained by Jones _et al_. (21). Using the same techniques, Simmons and Winzor (23), obtained eleven fractions from acetic acid dispersions of gluten with stepwise increases in the ionic strength to 0.2 M sodium chloride. Rechromatography of four of the fractions verified that these were definite chromatographic entities.

In recent years, gel-electophoretic techniques have been widely used in the study of gluten fractions. Woychik, Boundy, and Dimler (24) found nine components in aluminum lactate dispersion of gluten, using aluminum lactate buffer containing 3.0 M urea on starch gel. Gliadin showed eight components, and four had not been detected previously by the Tiselius method (21). The remaining high molecular weight component, glutenin, did not migrate in starch gel. Using the same technique, Coulson and Sim (25) found more than twenty components in acetic acid dispersion of gluten, and Elton and Ewart (26-28) detected eight components in acetic acid dispersions of gluten (26), and nineteen bands in the aluminum lactate dispersions of wet gluten obtained by hand-kneading flour in distilled water (27). Four of the major gliadin fractions have been recovered from

starch gel and have been found to give single bands when rerun (28). It was suggested that they are probably not formed by association reactions. Graham (29) studied the protein components in the extracts of wheat flour obtained with different solvents, and reported that similar components can be obtained but there were marked differences in the proportions of these components. Further studies on the fractions obtained by ion-exchange chromatography indicated that similar electrophoretic components are present in several of the fractions. A more complete study of fractionation of wheat flour on starch gel was reported by Kaminski (30). It was shown that twenty-one components were detected in aluminum lactate dispersions of gluten but only twelve were obtained after the previous removal of water-soluble and salt-soluble fractions of flour. Lee (31, 32) studied the acetic acid dispersions of gluten on polyacrylamide-gel and reported that fractions obtained according to the procedure of Simmons and Winzor (23) can be resolved into many bands. The detailed study of these fractions was reported recently by Lee _et_ _al_. (33). Seven fractions obtained by chromatography on carboxymethylcellulose were studied by sedimentation, gel-electrophoresis, chemical and N-terminal amino acid analysis. Sedimentation experiments indicated that four of these fractions were undergoing rapid, reversible association. N-terminal analysis and gel-electrophoresis showed that each fraction was grossly heterogeneous. More recently, Gehrke _et_ _al_. (34), using starch gel-urea electrophoretic technique to study the fractions obtained by chromatography, reported that gluten can be reasonably classified into six main classes, and, using

their experimental procedures, fairly pure fractions of these protein classes can be obtained by a single pass on a carboxymethylcellulose column.

Fractionation of acid-soluble proteins of gluten by partial solubility in methanol-chloroform mixture, and dilute formic aicd was reported by Meredith *et al*. (35). Five fractions varying in physical properties and amino acid composition were obtained. However, none of these fractions were electrophoretically homogeneous. Later, Meredith (36) attempted fractionations with three different solvent-precipitant systems; aqueous-salt solutions, dilute aqueous ammonia solutions, and methanol-chloroform solution. The first two solutions separated the gliadin from the nongliadin proteins, and the third fractionated the gliadin into two components (beta and gamma) by fractional precipitation with increasing concentrations of methanol.

Recently, gel-filtration chromatography has been successfully employed to fractionate proteins accoring to differences in molecular weight. Jones *et al*. (37) first applied this technique to separate wheat gluten protein on Sephadex G-75 columns. It was reported that sharp separations into gliadin and glutenin fractions as distinguished by gel electrphoresis were not achieved but that, ninety six percent electrophoretically pure glutenin, which is less soluble than that prepared by precipitation, can be obtained. Wright *et al*.(38) chromatographed wheat flour extracts on Sephadex G-100. They found that some of the gliadin proteins were not retarded on the columns, suggesting that species intermediate between gliadin and glutenin may exist. Further study was made by Beckwith *et al*.

(39) using the same techniques. They isolated and characterized a high molecular weight protein from the gliadin fraction. This protein resembled low molecular weight glutenin in its amino acid content, solubility characteristics, and mobility in moving-boundary and starch-gel electrophoresis.

The amino acid composition of gluten and its components.

The amino acid composition of gluten has been studied extensively since Osborne's time, owing both to the nutritional importance of specific acids and to the interest in characterizing the component proteins as related to the unique physicochemical properties of gluten.

The early studies on the amino acid composition of gluten reported results for only a limited number of amino acids. The difficulty lay not only in the complicated analytical methods, but also in the problems of hydrolysis of proteins contaminated with carbohydrate. The limitation as to types of samples studied, and amino acids determined, and the large variation in values reported, are evident in the results published from different laboratories. The first attempt to study the amino acid composition of the gliadin fraction was made by Abderhalden and Samuely (40) in 1905. Their results are comparable to Osborne's except for proline and glutamic acid. Osborne reported the amino acid composition of the various fractions of wheat in his monograph (1). Some of the results are quite close to the values determined by the more reliable column chromatography technique. Damodaran (41) and Stockelback and Bailey (42), both using Brazier's method (43) reported the amino acid composition of glutenin and gluten. The results of Damodaran are not very

different from those of Osborne. The results of Stockelback and Bailey, however, are quite different. Analytical methods gradually improved, and the results reported by Padoa (44) were closer to currently accepted values.

It was not until 1950 that the first complete amino acid analysis was made possible by the use of microbiological techniques. Using these techniques, Rice and Ramstad (45) determined the amino acid composition of wheat gluten. Only serine was undetermined. Following this, Pence et al. (46) reported the first complete amino acid analysis of wheat gluten. All amino acids except cystine and tryptophan were determined microbiologically. The results obtained in these two studies agree better with accepted values than did the earlier results.

The greatest advance in the quantitative determination of amino acids has been the application of chromatographic techniques. The reliability of these techniques has been clearly established (47). They not only provide the means for analyzing larger numbers of samples, but also require only small amounts of material. The first report on the amino acid composition of wheat gluten using this technique was made by Miller et al. (48) who used a starch column. Waldschmidt-leitz and Mindemann (49) also determined the amino acids of the glutelin fraction from barley, wheat, and rye using a starch column. Paper chromatography was used by Reznichenko et al. (50) in determining the amino acids of wheat and rye gliadins. After the automatic analysis of amino acids on ion-exchange columns was introduced by Stein and Moore (51), results for the amino acid composition of gluten and its fractions have been

frequently published. Woychik et al. (52) reported the amino acid composition of gluten and the various fractions obtained by electrophoretic separations. Meredith et al. (35) determined the amino acid composition of gluten fractions obtained by extracting with a methanol-chloroform mixture. Beckwith et al. (39) reported the amino acid composition of the higher and lower molecular weight protein fractions from gliadin. Some of these results will be referred to in the discussion section of this thesis.

The effect of reducing agents on gluten proteins.

A great deal of attention has been focussed on the dramatic effects of small amounts of oxidizing and reducing agents on bread doughs. Evidence showing that the reagents act directly upon the proteins was presented by many investigators (53-55) in the 1940's.

Extensive studies of the effect of reducing agents on gluten were made by Hlynka (56) and by Pence and Olcott (57). Hlynka added bisulfite, acetaldehyde, and similar reagents to the dough and then washed out the gluten. The results showed that the damage from bisulfite action to gluten can be reversed with acetaldehyde, and that only a restricted group of reducing agents has a bisulfite-like effect on dough. It was concluded that the changes in the gluten properties were probably due to the rupture of disulfide bonds or possibly the cross-linkages formed by carbonyl compounds. Hlynka (56) also pointed out that the reversibility of the effect by a simultaneous or later addition of acetaldehyde indicated a direct action on

the gluten and this was probably due to a specific reaction with disulfide groups,

$$RSSR + NaHSO_3 \rightleftharpoons RSH + RSSO_3Na,$$

rather than to mere reduction. Pence and Olcott have noted that the addition of monothioglycol to gluten dispersed in dilute acetic acid caused reduction in viscosity and decrease in cystine content, suggesting that the protein molecules are fragmented by the reducing agent.

Udy (58) studied the viscosity of wheat gluten dispersed in acetic acid and in 8% sodium salicylate before and after the addition of bisulfite and thioglycolic acid. It was reported that there are two major effects taking place when small quantities of either bisulfite or thioglycolic acid are added. The first is fairly complete repression of electrostatic effects and the second a complete reduction of all disulfide linkages.

Molecular weight studies on glutenin before and after reduction with sulfite or cleavage by performic acid oxidation were reported by Nielsen _et_ _al_. (59). It was shown that the reduction products give an ultracentrifugally homogeneous species with a molecular weight of 20,000. They concluded that glutenin contains basic polypeptide units with molecular weight of 20,000, and that various numbers of these units are linked via disulfide bonds to form the native proteins.

Reduced gliadin and glutenin were also studied by Woychik _et_ _al_. (60) using starch-gel electrophoresis. They reported that mercaptoethanol-reduced glutenin showed at least 10 major and 10 minor disulfide-linked compounds, whereas

reduced gliadin indicated no increase in number of major components. Some of the reduction components are common to both proteins. It was therefore suggested that extensive intermolecular disulfide bonding is responsible for the high molecular weight of glutenin and intermolecular disulfide bonding is present to only a limited extent in gliadin.

Beckwith *et al*. (61), studying the effect of disulfide bonds on molecular interactions of wheat gluten, reported that in urea solution below 5 M concentration only two-thirds of the disulfide bonds in gliadin are measured. Hydrogen bonding probably stabilizes the protein structure about the remaining disulfide bonds. They also indicated that thioethanol-reduced gliadin readily precipitates from urea solution at a lower salt concentration than gliadin, and reduced glutenin precipitates from urea solution at higher salt concentration than does native glutenin or either reduced or native gliadin.

Recently, Elton and Ewart (62) claim to have confirmed the hypothesis of Woychik *et al*. (60) that glutenin is built up chiefly from gliadin proteins by studying performic oxidized glutenin and gliadin. They reported that rupture of the glutenin caused a number of components to appear, some of which have mobility values equal to those of the corresponding ruptured gliadin.

The effect of proteolytic enzymes on gluten proteins.

Early investigations of the proteolytic enzymes on wheat gluten are numerous, but are largely concerned with the presence of such enzymes in wheat and flour, and their relationship to breadmaking and related processes. The

early literature on this subject was reviewed by Hildebrand (63) in 1946. The general view appeared to be that proteases are present in flour as well as in wheat, but the concentration of enzymes in normal flour is so low as to have little effect on gluten properties. The addition of excessive amounts of proteolytic enzymes will cause harmful effects in fermenting dough. However, the addition of appropriate quantities of the enzymes will improve bucky doughs.

The action of various proteolytic enzymes on gluten has also been studied extensively. Sharp and Elmer (64) found an increase in the amounts of protein soluble in 5% potassium sulfate, in 70% alcohol, and in the amounts of amino nitrogen as a result of autolytic digestion of flour. They concluded that gliadin was quite susceptible to the proteolytic enzymes of the flour whereas glutenin was not readily attacked. Harris (65) added various concentrations of different proteolytic enzymes to dough balls from which gluten was washed immediately and dispersed in 10% sodium salicylate solution. He found that, in the majority of instances, the inclusion of an enzyme hastened dispersion of the gluten, and this effect was found to increase with increasing enzyme concentration. Harris and Johnson (66) studied the effects of pepsin, papain, and potassium bromate upon viscosity and other properties of gluten dispersions. It was found that papain and pepsin have similar effects upon the rate at which gluten dispersed in 10% sodium salicylate and that bromate altered this rate. The quantity of protein precipitated from the gluten dispersion by magnesium sulfate was

markedly reduced by papain, but not by pepsin.

Swanson and Andrews (67) determined the amount of gluten which could be recovered from the doughs treated with various substances and found that the amount of gluten recovered from dough treated with papain decreases as the amount of papain was increased or the rest period was lengthened. This effect was not observed with the use of yeast water, cysteine, or glutathione. These results indicate that enzymatic disintegration of gluten took place in the dough.

Johnson and Miller and their associates (68-71) carried out a series of experiments on the effects of various enzyme preparations on doughs and on gluten dispersions. They suggested that the proteolytic enzymes break the peptide linkages and this results in increased extensibility of dough. Abbott, Miller, and Johnson (72) using a spectrophotometric method determined the extent of proteolysis of gluten and hemoglobin by various enzymes. They found that fungal and malted wheat flour proteinase attacked both hemoglobin and gluten in a similar manner while papain differed. Johnson, Miller, Boyer, and Geddes (73) have reported the action of proteases on gluten and hemoglobin under various conditions. They found that undenatured gluten was less readily hydrolyzed than hemoglobin, and that denatured gluten was even less susceptible to attack. The calculation of Michaelis-Menten constants and maximum velocity values showed that the rate of decomposition of the enzyme-substrate complex (Vm) is greater for hemoglobin than for

gluten. The affinity of the enzymes (Km) is greater for gluten than for hemoglobin. The apparent activation energies calculated for several enzyme systems were similar for hydrolysis of gluten, indicating that similar peptide bonds were hydrolyzed by the different protease systems acting on gluten. They also reported the optimum pH values for various enzymes acting on gluten. For malted wheat flour and fungal protease, the optimum pH was 3.0-4.0, for trypsin, it was 7.0-8.0, and for papain, it was near 5.5.

Strusi (74) studied the tryptic digests of glutens from durum and bread wheat. On circular paper chromatography, he observed that there were considerable differences in the content of free amino acids and peptides in digestion mixtures of the two, although the amino acid compositions of the two glutens were similar. It was suggested that the differences in the glutens may lie in their amino acid sequences in the gluten molecules.

Rohrlich and Schulz (75) studied the peptic digests of zwickelprotein, haftprotein, and gliadin of wheat. Using ion-exchange chromatography, they found a similar peak appeared in each chromatogram. Amino acid analyses indicated that they were made up of similar amino acids. It was therefore concluded that there is a partial similarity in structure of these proteins.

Finlayson (76) studied gliadin hydrolyzed by treatment with pepsin and then by trypsin. It was shown that a large number of peptides were produced as well as small amounts of some amino acids. Five peptides were obtained crystalline

and two of these constituted 11.5% and 1.2% of gliadin, respectively. Their structures have been partly determined. The results indicate that wheat gliadin contains (Asp-Asp) bonds and at least 1.9% of the glutamic acid is present as glutamylglutamic acid.

The action of pepsin on glutenin has recently been studied by Oka _et_ _al_. (77). They reported that pepsin rapidly decreases the viscosity of a glutenin dispersion and rapidly increases the amount of water-soluble fragments. A comparison of data obtained by gel filtration and by determination of terminal amino acids in pepsin hydrolyzates of glutenin indicates that cleavage of a few peptide bonds rapidly produces these large molecular polypeptides from glutenin. Further pepsin action causes a substantial increase in small peptide fragments.

Verma and McCalla (78) studied papain and peptic digests of gluten. It was shown that there were qualitative, and probably quantitative differences in the action of enzymes. Precipitates formed in all digests of sodium salicylate dispersions, and the amino acid distribution in these precipitates was quite different from that of whole gluten.

More recently, Ewart (79) reported the application of fingerprinting to glutenin and gliadin. It was shown that there may be a basic similarity in the amino acid sequences of considerable portions of the polypeptide chains present in glutenin, and gliadin. He supported the idea of Woychik _et_ _al_. (60) that glutenin is synthesised from gliadin constituents.

MATERIALS AND METHODS

The flour used as the starting material for the preparation of gluten was from Marquis wheat, milled on an experimental Bühler mill, and extracted with ether. The protein content of the ether-extracted flour was 16.25% (N x 5.7) on the dry basis.

All chemicals used in this work were reagent grade, except that aluminum lactate was prepared according to the method described by Jones and Cluskey (80). The enzymes were obtained from the following companies: Crystalline pepsin, 3x, from Calbiochem Company; Crystalline trypsin from C. F. Boehringer and Soehne, GmbH Mannheim; and Papain from Difco Laboratories. The resin, Aminex 50W-X2, was obtained from Calbiochem Company.

Preparation of gluten and gluten dispersions.

Gluten was washed from the flour using a gluten-washing machine* and o.1% phosphate buffer of pH 6.8, prepared according to the method of Dill and Alsberg (81).

Gluten from 10 g of flour was dispersed in 50 ml of 10% sodium salicylate with or without 25 mg of added sodium bisulfite, or in 100 ml of 0.02 M aluminum lactate buffer of pH 3.10. Dispersion was carried out in a 125 ml glass-stoppered flask with occasional stirring. After all gluten had dispersed (24 hours), the starch and non-gluten materials were removed by centrifugation at 12,000 g for 20 minutes using an angle centrifuge at 3°C. The supernatant was decanted and dialyzed against the appropriate dispersing

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* Theby, Ertel-Werk, Munchen.

agent at 3°C for 24 hours. The nitrogen concentration of each of the final dispersions was 4.0 mg/ml in 10% sodium salicylate solution with or without added bisulfite, and 2.1 mg/ml in 0.02 M aluminum lactate buffer.

Fractionation of dispersed gluten.

Gluten dispersions in 10% sodium salicylate solution, with or without added bisulfite or sulfite, were fractionated essentially according to the procedure of McCalla and Rose (11) by successive addition of calculated amounts of a saturated magnesium sulfate solution. The saturated magnesium sulfate solution was slowly added from a burette to the dispersion which was kept constantly stirred to avoid local excesses. After the addition of the salt, the dispersions were stored at 3°C overnight. The precipitated proteins were then removed by centrifugation at 12,000 g for 20 minutes,the supernatant was decanted, and the proteins were redispersed in 10% sodium salicylate solution in the presence of bisulfite. The process was repeated with increasing amounts of magnesium sulfate being added to the supernatant to obtain additional fractions. Seven fractions were obtained from the dispersion without added bisulfite, and designated Fractions I, II, III, IV, V, VI, and VII, respectively for the proteins precipitated at salt concentrations of 0.025, 0.04, 0.06, 0.08, 0.10, 0.20, and 0.50 of saturation. Six fractions were obtained from the dispersion with added bisulfite, and designated Fractions IIB, IIIB, IVB, VB, VIB, and VIIB, respectively for the proteins precipitated at the salt concentrations of 0.04, 0.06,

0.08, 0.10, 0.20, and 0.50 of saturation. Six fractions were obtained from the dispersion with added sulfite, and designated Fractions IIS, IIIS, IVS, VS, VIS, and VIIS, respectively for the proteins precipitated at the salt concentrations of 0.04, 0.06, 0.08, 0.10, 0.20 and 0.50 of saturation. Redispersion of precipitated protein was accomplished with no difficulty except that Fractions I and IIB were redispersed by using an Omni mixer, blending at a low speed for 5 minutes.

The fractionation of gluten dispersed in aluminum lactate was carried out by the successive additions of 0.5 N sodium hydroxide solution to the dispersion which was stirred constantly. When the desired pH was reached, the dispersion was mixed thoroughly and stored overnight at 3°C. It was then centrifuged at 12,000 g for 20 minutes. The supernatant was decanted, and the precipitate was redispersed in 0.02 M aluminum lactate buffer, pH 3.10. Further fractions were obtained by repeated addition of 0.5 N sodium hydroxide to the supernatant. The final supernatant was dialyzed against 0.01 M acetic acid for two days at 3°C. It was then freeze-dried, and redispersed in 0.02 M aluminum lactate buffer at pH 3.10. Fractions were obtained by precipitation at pH values of 4.00, 4.40, 4.80, and from the supernatant at pH 4.80, and were designated respectively Fractions A, B, C, and D.

Determination of amino acids.

The chromatographic analysis of amino acids was carried out on a Spinco amino acid analyzer, using columns packed with Beckman type AA-15 and AA-27 resins and operated at the flow rate of 68 ml/hour. Potassium cyanide was used as the catalyst at the concentration of 0.00004 N in the eluting buffer for the color development of amino acids with ninhydrin reagent, which was pumped at the flow rate of 34 ml/hour, and was prepared by dissolving 80 g ninhydrin in 4 liters of 4 N sodium propionate buffer pH 5.5 and methyl cellosolve 1:3 mixture.

The samples for chromatographic analysis of amino acids were prepared in the following manner:

An aliquot of the fraction was hydrolyzed by refluxing with 30 ml of constant boiling hydrochloric acid for 24 hours. The hydrolyzate was evaporated to dryness at 50°C on a rotary evaporator. The dried hydrolyzate was taken up in 100 ml of demineralized water and evaporated to dryness again. It was then freeze-dried to remove water completely, and dissolved in a known volume of sodium citrate buffer of pH 2.2 to make about 0.2 to 0.3 mg N/ml concentration. The solution was filtered and one ml was used for the determination of the acidic amino acids, and two ml for the basic amino acids. In addition, part of each solution was diluted to about 0.05 to 0.06 mg N/ml for the determination of glutamic acid and ammonia. (This was necessary because of the high content of these substances). A blank was run in exactly the same manner for the correction of ammonia content.

For the determination of tryptophan, an aliquot was hydrolyzed by refluxing with 10 ml of 5 N sodium hydroxide for 20 hours in an alkali-resistant flask. The hydrolysate was neutralized with dry ice and filtered. The filtrate was evaporated to dryness on a rotary evaporator at 50°C. The dried sample was then taken up to a desired concentration with sodium citrate buffer of pH 2.2. One ml of the sample was transferred to the short column, the length of which had been increased to 11 cm to obtain better resolution. Tryptophan was eluted just before lysine.

Enzymic digestion of gluten.

The enzymic digestions were performed in Erlenmeyer flasks at 37.5°C using suitable proteolytic enzymes. Papain and trypsin were used for the gluten dispersed in sodium salicylate, and pepsin was used for the gluten dispersed in aluminum lactate. The concentration of each enzyme was kept at 1% by weight of the protein used. At appropriate intervals aliquots were withdrawn and the undigested and partially digested proteins were precipitated with one fifth volumes of 50% trichloroacetic acid. The solutions were centrifuged and the protein-free supernatants were acidified with hydrochloric acid and extracted with ether to remove the remaining salicylic acid and trichloroacetic acid which might interfere with the subsequent analysis. After the digestion was concluded, the protein precipitated during digestion was removed by centrifugation. Digestion was stopped by freezing the digestion mixture and storing at -20°C until used for

further study. The precipitated proteins were dissolved in 0.1 N sodium hydroxide and aliquots of the solutions were hydrolyzed with 6 N hydrochloric acid for amino acid analysis.

Determination of non-protein nitrogen, terminal amino group nitrogen, and free amino acid nitrogen.

Aliquots of the protein-free supernatants prepared as described above, were used for the following determinations.

Non-protein nitrogen was determined by the microKjeldahl method using mercuric oxide and potassium sulfate as catalysts(99). Terminal amino group nitrogen was determined using Fisher's Van Slyke apparatus. Free amino acid nitrogen was determined by the Conway microdiffusion method as described by McConnell (82).

Ion exchange chromatography of digests.

The digestion mixtures were chromatographed at 38°C by gradient elution on a 150 x 0.9 cm column of Aminex 50W-X2 with 200-325 mesh. Before the experiments, the resin was stirred and washed several times with 2 N sodium hydroxide in a fritted funnel and then equilibrated resin was settled several times from the buffer in order to remove the very fine particles. The resultant resin was packed into the column by using a pump with the flow rate of 60 ml/hour.

When the digests were to be employed for chromatographic separations, the stored digest, having been at neutral pH, was brought to pH 2.5 by the addition of 1 N acetic acid. The salicylic acid formed was centrifuged off, and the supernatant was freeze-dried. The dried sample was taken up in a small

amount of sodium citrate buffer of pH 2.2.

The sample was applied on the top of the column and forced in with nitrogen gas pressure. The elution was begun with 0.2 N buffer at pH 3.10 at the flow rate of 30 ml/hour, and ionic strength and pH of the buffer were gradually changed to 2.0 N and pH 6.8 by using a nine chamber Autograd, which was filled with the buffers made up according to Catravas (83) as given in Tables I and II.

TABLE I

Composition of Buffers used for Peptide Analysis

	Eluent buffers		
pH	3.10	5.10	6.80
Na conc.	0.2 N	2.0 N	2.0 N
Citric acid-H_2O	21.0 g	105.0 g	-
NaOH (97%)	8.3 g	47.06 g	-
Conc. HCl	18 ml	-	-
Glacial acetic acid	-	21.47 ml	-
Sodium acetate-$3H_2O$	-	-	272.17 g
Final vol.	1 l.	1 l.	1 l.

TABLE II

Buffer Composition of Autograd Chambers

Chamber No.	Buffers, ml			Water, ml	0.01 N NaCN*,ml
	3.10	5.10	6.80		
1-4	92	-	-	-	0.3
5	57	15	-	20	0.3
6	4	45	-	40	0.3
7	-	88	-	-	0.3
8	-	67	20	-	0.3
9	-	-	87	-	0.3

*NaCN solution was added to the chambers just before the start of the run.

The eluent containing peptides was mixed with ninhydrin reagent for color development. This reagent, pumped at the rate of 34 ml/hour, was composed of two volumes of ninhydrin reagent (used for the amino acid analysis) and one volume of distilled water. The color development was carried out at 100°C for approximately 7 minutes. The optical density of the solution was measured and recorded automatically in the same manner used for amino acids. The temperature of the column was increased to 55°C after being held for a period of 20 hours at 38°C. The rate was maintained constant throughout the experiment. Peptides generally react with ninhydrin only at a free amino group, so this procedure measures only the free amino group of the peptides.

If a peptide is hydrolyzed then all the liberated amino acids will react with ninhydrin. Consequently, in some experiments, a fraction collector* was used, and the fractions were hydrolyzed, then tested with ninhydrin.

The hydrolysis of peptides was carried out essentially according to the method of Hirs _et al_. (84) as follows:

o.3 ml aliquots placed in a set of Pyrex test-tubes (10 x 100 mm) were hydrolyzed with 1.0 ml of 2.5 N sodium hydroxide in a water bath at 95°C for 2.5 hours. After hydrolysis, the tubes were cooled and then to each of the cooled tubes, 1.0 ml of 30% (by volume) acetic acid was added to bring the final solution to about pH 5. The final solution was mixed thoroughly with 1 ml ninhydrin and heated at 100°C for 10 minutes. After the solution cooled, the optical density was measured at 570 mμ with a photometer.

Determination of ribonucleic acid.

The hydrolysis of total RNA was based in principle on the Schmidt and Thannhauser method (85).

The precipitates from enzymic digests were dissolved in 15 ml of 0.5 N potassium hydroxide and hydrolyzed for 18 hours at 37°C. The solutions were then cooled to 0°C and made slightly acid by dropwise addition of cold 7 N perchloric acid (PCA). The hydrolyzates were separated by centrifugation in the cold and the residue washed 3 times with 0.3 N PCA. The combined washings and hydrolyzate, after neutralization with 7 N KOH, stood overnight in the cold.

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*Model 132, Fraction Collector, Beckman.

The precipitated potassium perchlorate was removed by centrifugation, and the supernatant was evaporated to dryness on a rotary evaporator at room temperature.

The dry residues containing the mononucleotides, were dissolved in 5 ml of distilled water and deproteinized by shaking with a chloroform-octanol mixture using a vortex mixer. The solutions were then centrifuged at 11,000 g and the chloroform layer siphoned off. This process was repeated at least 5 times to make sure that all the protein was removed. The final aqueous layer was used for the estimation of nucleotide concentration.

The separation of the mononucleotides was carried out using a paper chromatographic technique which is essentially that of Lane (86). 100 microliter portions of the solutions were spotted in a band on a paper which was impregnated with ammonium sulfate by dipping the paper into a one-tenth saturated solution and allowing the excess liquid to drip off. The chromatograms were developed for 22-26 hours by the descending method using 80% ethanol in a 12 x 12 x 24 inch all glass tank which was pre-saturated with the developing solvent.

After development, the chromatograms were removed and air dried. The nucleotides were located by scanning with a short wave, Mineralight*. The spots were cut off along with a blank, and the paper discs were then cut into small bits and disintegrated on a vortex mixer with 5 ml of 0.12 N HCl. The paper was removed by centrifugation at 10,000 g and the

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*Model UVS 11, Ultra-violet Products, Inc., San Gabriel, Cal.

optical density of the supernatant measured between 300 and 230 mμ in the Beckman DK-1 spectrophotometer. From the spectral data the nucleotide concentration was calculated using the following molar extinction coefficient at 260 mμ (E_{260}) in acid:

Cytidylic acid	6.8×10^3
Guanylic acid	11.8×10^3
Adenylic acid	14.2×10^3
Uridylic acid	10.0×10^3.

RESULTS

Fractionation of dispersed gluten from 10% sodium salicylate solution with or without added bisulfite or sulfite.

It had been noted during the sedimentation study of gluten (87) that the addition of small quantities of bisulfite did not change sedimentation diagrams significantly. It seems possible that the action of bisulfite causes merely a physical disintegration of aggregated gluten rather than reduction of disulfide bonds. On the other hand, the addition of sulfite has been shown to reduce the sedimentation constant of dispersed gluten (88) and to break disulfide bonds (59). Therefore, bisulfite and sulfite were used as reducing agents in the present investigation.

Fractionation of dispersed gluten. This experiment was conducted to determine the quantities of proteins precipitated at certain concentrations of added bisulfite or sulfite.

Four gluten dispersions were prepared in the usual manner in 10% sodium salicylate solution containing the following amounts of sodium bisulfite: 0, 10, 25, and 50 mg/50 ml dispersion. Two further gluten dispersions were prepared in 10% sodium salicylate solution, one containing 25 mg/50 ml of sodium bisulfite and the other 25 mg/50 ml of sodium sulfite, and each was dialyzed for 24 hours against a large amount of the appropriate dispersing agent in order to ensure that the full effects of the reagents would be obtained.

The final dispersions prepared as described above were different in opacity. The first four were opaque and slightly yellowish, whereas the other two were slightly translucent. All of these dispersions were then fractionated. The relation between nitrogen precipitated and the concentration of the precipitating reagent is shown in Figure 1, each point being an average of duplicate determinations. The reproducibility of the results was within 3 to 8%, depending upon the fractions. Since the first two fractions were sensitive to very small amounts of magnesium sulfate, a slight change in the amount added or in the degree of saturation of the precipitating agent would cause considerable variations in these fractions. However, slight variations in the proportions of fractions would not significantly affect the relation of nitrogen recovery and the concentration of magnesium sulfate.

It is evident from Figure 1, that there were no significant changes in the quantitative values of the fractions when small quantities of bisulfite were added, since the basic curve in Figure 1 is a composite of results from four different dispersions, and there are no detectable differences in the curves. The precipitation of protein started at o.025 saturation of the dispersion with magnesium sulfate, and progressively increased with no indication of discontinuity. This is in accord with the results obtained by McCalla and Rose (11). After dialysis in the presence of bisulfite, however, the precipitation started at 0.035 saturation, and increased nearly linearly up to 70% precipitation of the

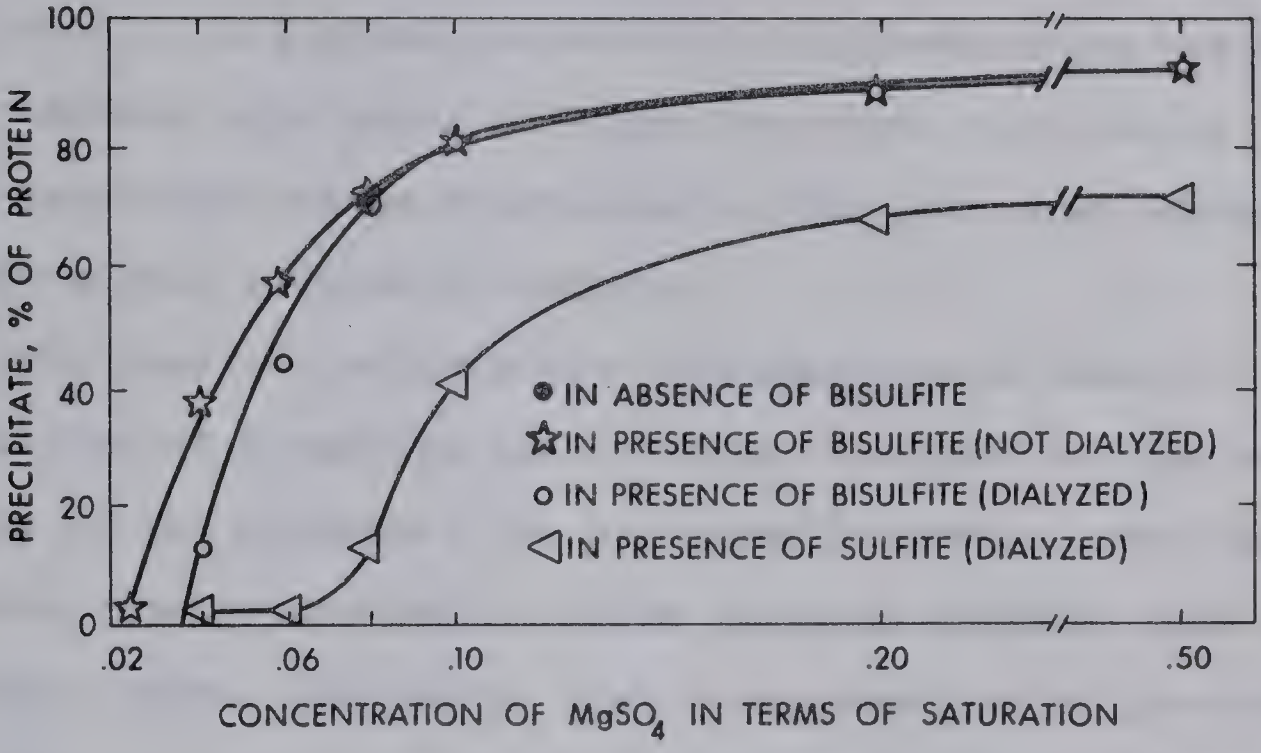


FIGURE 1. Precipitation of dispersed gluten with $MgSO_4$.

protein, then followed the curve for the dispersion with no added bisulfite. With the dispersion dialysed against sulfite, a small amount of protein precipitated at 0.04 saturation, but no further precipitation occurred until 0.075 saturation. Precipitation progressed regularly with the addition of increasing amounts of magnesium sulfate, but the total of all precipitates at 0.5 of saturation was only 72% compared with about 95% from the other dispersions. This variation can be attributed to the increased solubility of the gluten reduced by sulfite.

The results indicate that the addition of small quantities of bisulfite has no apparent effect on the solubility of the proteins. On prolonged treatment with bisulfite, however, the precipitation curve deviated slightly from the original curve, indicating that disaggregation of proteins or rupture of some of the disulfide bonds probably occurred. On prolonged treatment with sulfite, the precipitation curve was drastically changed, probably indicating that extensive cleavage of the disulfide linkages occurred. The discontinuity in this curve at the initial stage indicates that a small portion of the proteins may not be reduced by sulfite.

Analysis of the fractions. Having shown by the preceding experiments that there was a considerable difference in the distribution of fractions after gluten dispersions had been subjected to prolonged treatment with bisulfite or sulfite, it was decided to determine the amino acid composition of these fractions.

The results are presented in Tables III, IV, and V. Reported values were generally based on the results of separate analysis of two samples. In most cases, the agreement between values was good, the variation being generally less than 3%. With certain amino acids, namely cystine, methionine, serine, and threonine, however, which were present in low concentration, or unstable in the acid, the variation was beyond this limit.

The results presented in Table III were obtained by analysis of the fractions prepared from gluten dispersed in 10% sodium salicylate without added bisulfite. Comparison of the values shows that the less soluble proteins generally contain a higher proportion of lysine, arginine, aspartic acid, threonine, glycine, alanine, and tryptophan, and a slightly higher proportion of serine, valine, methionine, leucine, and tyrosine, but lesser amounts of glutamic acid, proline, and phenylalanine. The amounts of histidine, cystine, and isoleucine, are nearly constant. The amide-nitrogen content, as measured by the ammonia, increases as the solubility of proteins increases. In most instances, the changes in amino acid content were regular, there being no break from Fractions I to V. This is clearly indicated when the values are plotted at the cumulative mid-points of the fraction as presented in Figure 2, the mid-points being expressed as percentages of the total nitrogen in the original dispersion. It is obvious from Figure 2, that, except for the most soluble 10 to 15% of the protein, most of the amino acid values increase or

TABLE III

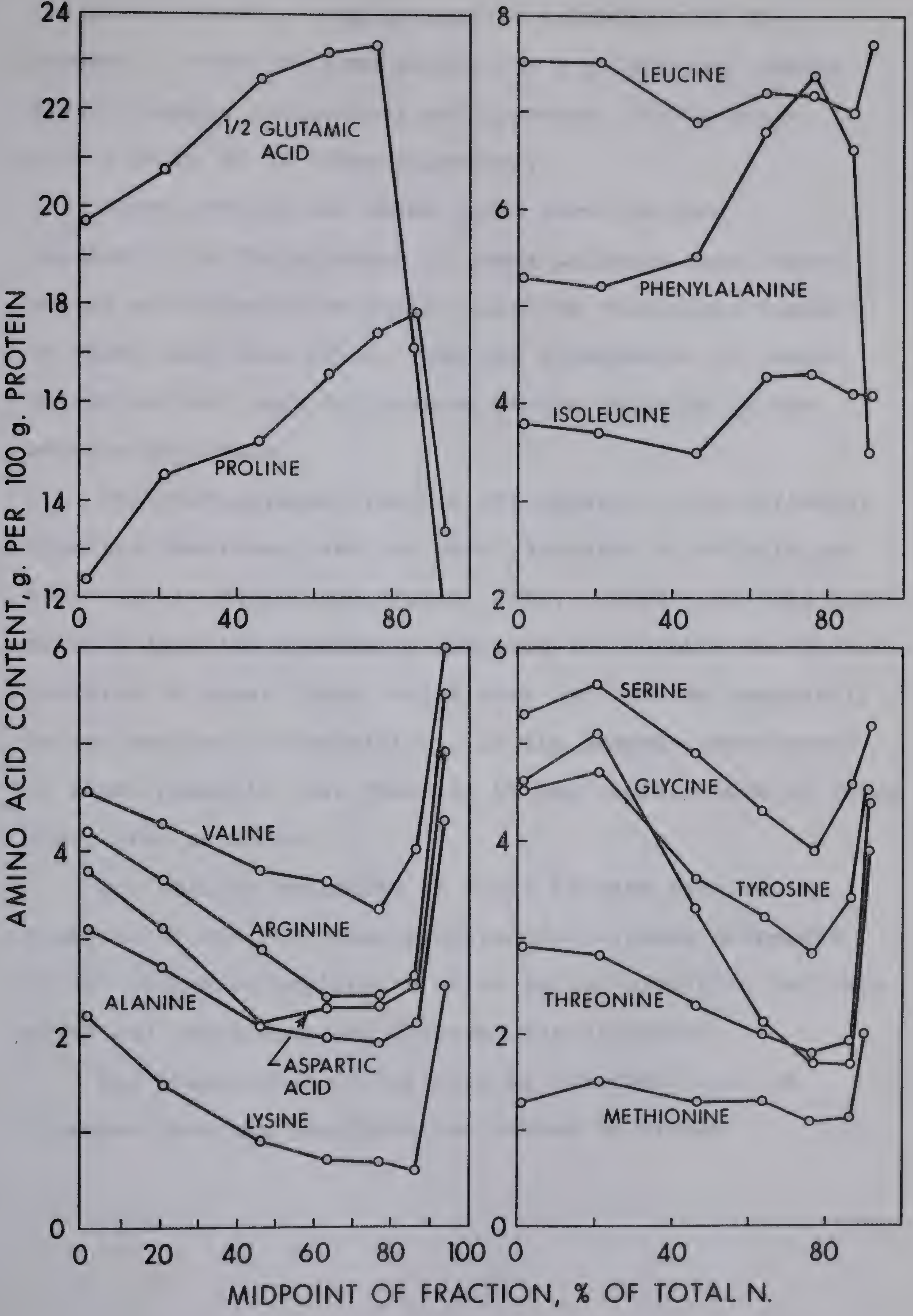
The amino acid composition of gluten and its fractions obtained from gluten dispersed in 10% sodium salicylate without added bisulfite.

Fractions	Gluten	I	II	III	IV	V	VI	VII	
Lysine	1.1	2.2	1.5	0.9	0.7	0.7	0.6	2.5	
Histidine	2.0	2.3	2.1	2.0	2.0	2.0	2.2	2.7	
Ammonia	5.3	4.8	4.9	5.6	5.9	5.8	4.6	3.0	
Arginine	3.0	4.1	3.6	2.9	2.4	2.3	2.5	6.0	
Aspartic acid	2.7	3.7	3.1	2.1	2.3	2.4	2.6	4.9	
Threonine	2.6	2.9	2.8	2.3	2.0	1.8	1.9	4.5	
Serine	4.8	5.3	5.6	4.9	4.3	3.9	4.6	5.2	
Glutamic acid	42.0	39.3	41.3	45.2	46.2	46.5	34.2	26.6	
Proline	15.0	12.4	14.5	15.2	16.5	17.4	17.8	10.5	
Glycine	3.2	4.6	5.1	3.3	2.1	1.7	1.7	3.9	
Alanine	2.3	3.1	2.7	2.1	2.0	1.9	2.1	4.2	
Cystine	2.0	1.4	1.8	1.8	1.5	1.7	1.3	3.5	
Valine	3.8	4.5	4.2	3.7	3.6	3.3	3.9	5.5	
Methionine	1.3	1.3	1.5	1.3	1.3	1.1	1.1	2.0	
Isoleucine	3.7	3.8	3.7	3.5	4.3	4.3	4.1	4.1	
Leucine	6.8	7.5	7.5	6.9	7.2	7.2	7.0	7.7	
Tyrosine	3.7	4.5	4.7	3.6	3.2	2.8	3.4	4.4	
Phenylalanine	5.6	5.3	5.2	5.5	6.8	7.4	6.6	3.5	
Tryptophan	2.0	5.6	2.6	0.9	1.0	1.0	1.3	4.9	
N cal/N deter %	95.3	99.8	99.5	99.5	99.3	99.5	88.9	89.5	Recovery
% of total proteins	-	4.4	32.1	19.8	15.0	10.0	11.4	2.7	95.4

All values reported as grams amino acid/17.5 grams nitrogen.

FIGURE 2.

Amino acid content of gluten fractions obtained from gluten dispersed in 10% sodium salicylate without added bisulfite.


AMINO ACID CONTENT, g. PER 100 g. PROTEIN
MIDPOINT OF FRACTION, % OF TOTAL N.
1/2 GLUTAMIC ACID
PROLINE
LEUCINE
PHENYLALANINE
ISOLEUCINE
VALINE
ARGININE
ALANINE
ASPARTIC ACID
LYSINE
SERINE
GLYCINE
TYROSINE
THREONINE
METHIONINE
24
22
20
18
16
14
12
8
6
4
2
0
0
20
40
60
80
100

decrease smoothly with increasing solubility of the protein. There are some exceptions e.g. glycine, serine, phenylalanine, isoleucine, and tyrosine, show a break at the point of 20 % precipitation.

Since not all the amino acids show the same regularity in distribution it seems unlikely that interaction of two proteins could yield the variations found. It seems much more likely that the differences in composition reflect real differences in the proteins in the various fractions.

The most soluble Fraction VII appears to be different from the remainder, and has been discussed by McCalla and Rose (11) in their experiments. They stated that this most soluble fraction apparently contains the soluble non-gluten proteins of wheat flour, which were not removed completely during washing of the gluten. In the present experiments it seems possible that Fraction VI may contain some of these non-gluten proteins.

The results presented in Table IV were obtained by analysis of the fractions prepared from gluten dispersed in 10% sodium salicylate to which sodium bisulfite had been added and the dispersion dialyzed for 24 hours.

The proportions of the various fractions were as expected from the precipitation curves in Figure 1.

TABLE IV

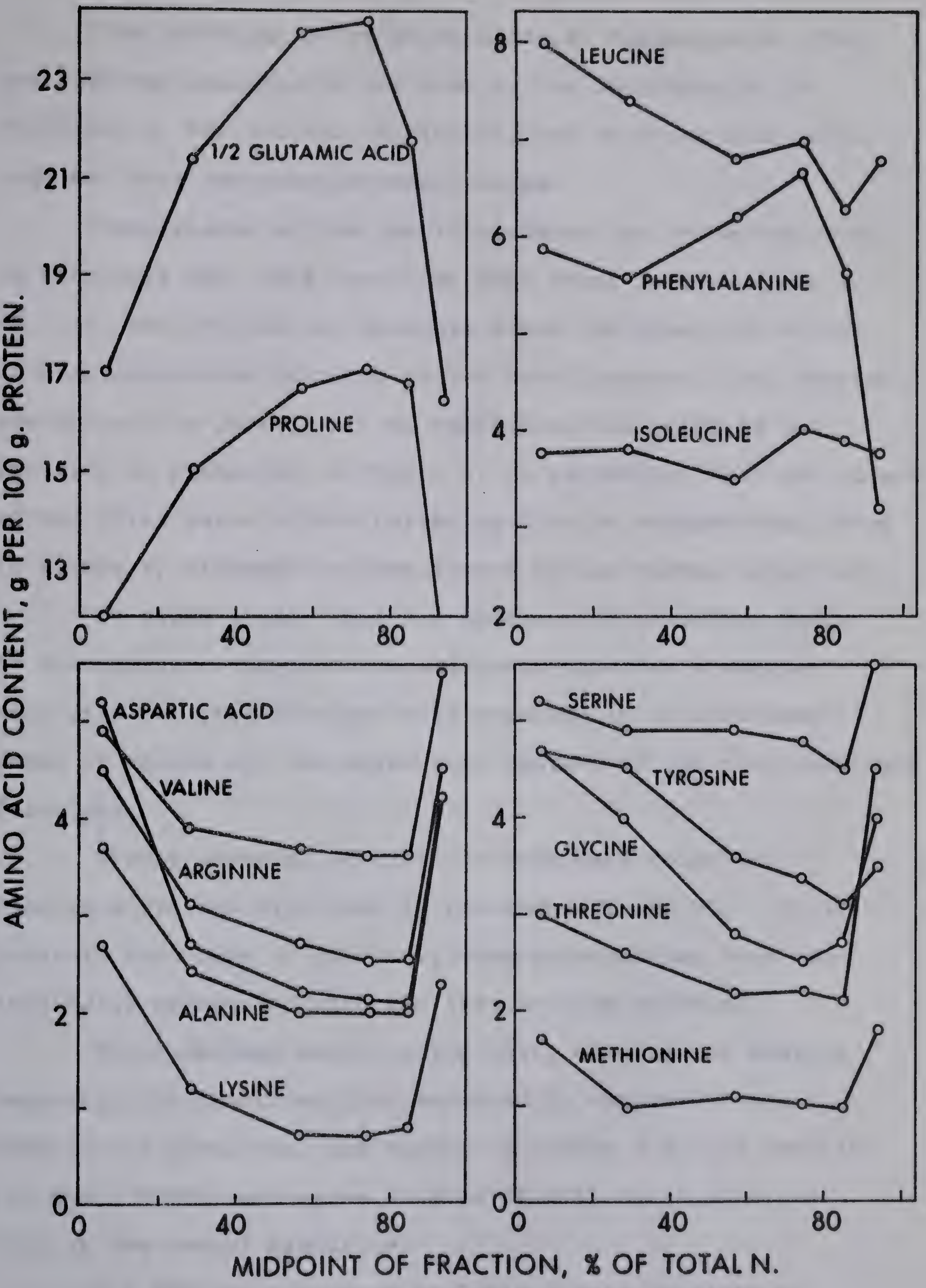
The amino acid composition of gluten dispersed in 10% sodium salicylate solution with added bisulfite, and of its fractions obtained by the addition of magnesium sulfate

Fractions	Gluten	IIB	IIIB	IVB	VB	VIB	VIIB	
Lysine	1.4	2.7	1.2	0.7	0.7	0.8	2.3	
Histidine	2.2	2.4	2.2	2.0	2.0	1.9	2.7	
Ammonia	5.5	4.5	5.2	6.0	6.1	5.3	3.8	
Arginine	3.3	4.5	3.1	2.7	2.5	2.5	5.5	
Aspartic acid	3.0	5.2	2.7	2.2	2.1	2.1	4.5	
Threonine	2.9	3.0	2.6	2.2	2.2	2.1	4.5	
Serine	5.1	5.2	4.9	4.9	4.8	4.5	5.6	
Glutamic acid	43.2	33.9	42.8	48.0	48.5	43.6	32.8	
Proline	15.3	12.0	15.0	16.7	17.1	16.8	10.4	
Glycine	3.5	4.7	4.0	2.8	2.5	2.7	4.0	
Alanine	2.4	3.7	2.4	2.0	2.0	2.0	4.2	
Cystine	1.4	2.1	0.8	1.3	0.2	0.3	0.9	
Valine	4.0	4.9	3.9	3.7	3.6	3.5	5.5	
Methionine	1.3	1.7	1.0	1.1	1.0	1.0	1.8	
Isoleucine	3.8	3.8	3.8	3.5	4.0	3.9	3.8	
Leucine	7.3	8.0	7.4	6.8	7.0	6.3	6.8	
Tyrosine	4.1	4.7	4.5	3.6	3.4	3.1	3.5	
Phenylalanine	5.9	5.9	5.6	6.2	6.7	5.6	3.2	
Tryptophan	2.1	7.6	2.0	1.2	0.9	1.4	4.4	
N cal/N deter,%	96.3	99.8	99.8	99.5	99.8	99.5	97.3	Recovery
% of total Proteins		11.5	31.9	25.5	12.0	10.0	4.0	94.9

All values reported as grams amino acid/17.5 grams nitrogen.

FIGURE 3

Amino acid content of gluten fractions obtained from gluten dispersed in 10% sodium salicylate after prolonged treatment with bisulfite.


AMINO ACID CONTENT, g PER 100 g. PROTEIN.
MIDPOINT OF FRACTION, % OF TOTAL N.
1/2 GLUTAMIC ACID
PROLINE
23
21
19
17
15
13
LEUCINE
PHENYLALANINE
ISOLEUCINE
8
6
4
2
ASPARTIC ACID
VALINE
ARGININE
ALANINE
LYSINE
SERINE
TYROSINE
GLYCINE
THREONINE
METHIONINE
4
2
0
0
40
80

The distribution of amino acids in Fractions VB, VIB, and VIIB was essentially the same as the distribution in Fractions V, VI, and VII (Table III) and this was also to be expected from the precipitation curves.

Comparisons of the specific values for the amino acids in Fractions IIB, IIIB, and IVB, with those for Fractions I, II, III, and IV, are not possible since the fractions do not include comparable portions of the total protein. If, however, the values are plotted at the cumulative mid-point of the fraction as presented in Figure 3, it is obvious that the slopes of the first parts of the curves tend to be steeper than those in Figure 2, although the remainders of the curves agree well.

It seems likely that the addition of bisulfite tends to disaggregate the proteins and hence provides a sharper separation of fractions and this resulted in an increased range of values for the amino acid content of the most insoluble fractions.

Higher contents of glutamic acid were found in Fractions VIB and VIIB than in Fractions VI and VII. It is possible that some of the non-gluten proteins may have been originally aggregated with the less soluble proteins.

It is perhaps worth noting that, despite the sharper separation of fractions that appeared to occur with the addition of bisulfite, the curves in Figure 3 do not exhibit any sharp break, and agree in general with those obtained without the use of bisulfite.

The results presented in Table V were obtained by analysis of the fractions prepared from the gluten dispersion

after it was reduced with sulfite.

An examination of the precipitation curves in Figure 1 shows that after general precipitation began the curve for the sulfite-treated gluten roughly parallels the curve for the untreated and bisulfite-treated samples. Similarly, the analytical results for Fractions VS and VIS presented in Table V and Figure 4, are similar to those for IVB, VB, and VIB (Table IV). Fractions VS and VIS together make up 55.8% of the total protein in the dispersion, while Fractions IVB, VB, and VIB make up 47.5% of the total in that dispersion. Fractions IIS and IVS correspond roughly in quantity and amino acid composition to Fraction IIB and in composition also to Fraction I. There is, in the sulfite-treated gluten, however, much less of the total gluten with amino acid distribution similar to that found with the less soluble fractions of the other dispersions. Thus there is no fraction corresponding to Fraction II or to Fraction IIIB. Each of these fractions makes up about 30% of the total protein of the dispersions and this is approximately the proportion that is not recovered by precipitation after sulfite treatment.

It therefore appears that the sulfite reacted with the less soluble fractions, probably splitting intermolecular disulfide bonds. If sulfite split disulfide bonds of the more soluble fractions, these must have been intramolecular bonds since there was no apparent change in solubility. Further comments on these results will be made in the discussion section of the thesis.

TABLE V

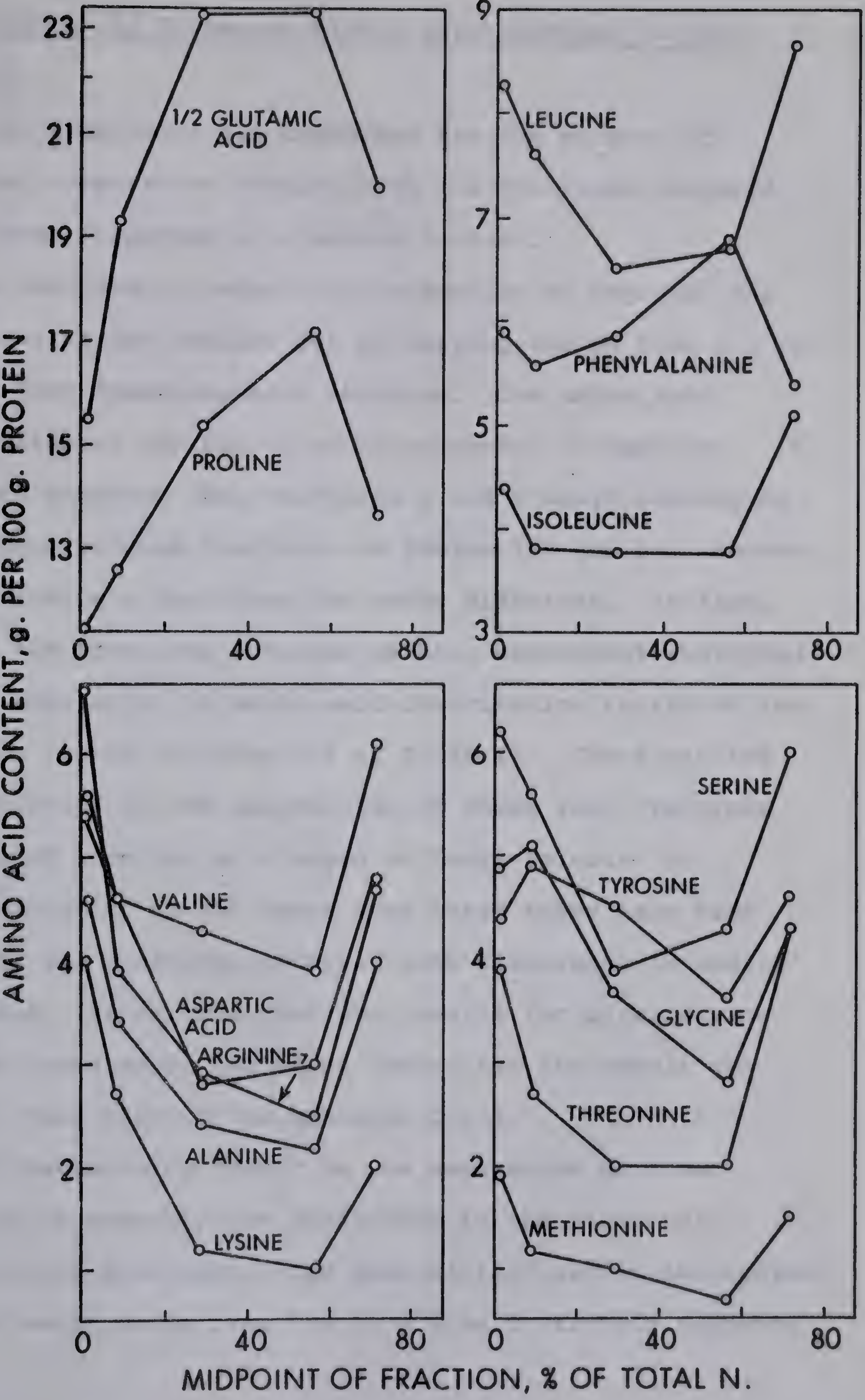
The amino acid composition of the fractions obtained from gluten dispersed in 10% sodium salicylate solution after prolonged treatment with sulfite.

Fractions	IIS	IVS	VS	VIS	VIIS	
Lysine	4.0	2.7	1.2	1.0	2.0	
Histidine	2.6	2.4	2.4	2.3	2.9	
Ammonia	3.8	4.6	5.6	5.5	3.3	
Arginine	5.4	4.6	2.8	3.0	4.7	
Aspartic acid	6.6	3.9	2.9	2.5	4.8	
Threonine	3.9	2.7	2.5	2.5	4.3	
Serine	6.2	5.6	3.9	4.3	6.0	
Glutamic acid	30.9	38.6	46.4	46.5	40.0	
Proline	11.5	12.6	15.4	17.2	13.7	
Glycine	4.9	5.1	3.7	2.8	4.3	
Alanine	4.6	3.4	2.4	2.2	4.0	
Cystine	2.0	1.5	0.8	1.2	1.9	
Valine	5.6	4.6	4.3	3.9	6.1	
Methionine	1.9	1.2	1.0	0.7	1.5	
Isoleucine	4.4	3.8	3.8	3.8	5.1	
Leucine	8.4	7.6	6.5	6.7	8.9	
Tyrosine	4.4	4.9	4.5	3.6	4.6	
Phenylalanine	5.9	5.6	5.9	6.8	5.4	
N cal/N deter, %	99.0	99.0	98.0	98.0	98.0	Recovery
% of total proteins	1.4	13.7	26.0	29.8	0.5	71.4

All values reported as grams amino acid/17.5 grams nitrogen.

FIGURE 4.

Amino acid content of gluten fractions obtained from gluten dispersed in 10% sodium salicylate after prolonged treatment with sulfite.


AMINO ACID CONTENT, g. PER 100 g. PROTEIN
MIDPOINT OF FRACTION, % OF TOTAL N.
1/2 GLUTAMIC ACID
PROLINE
23
21
19
17
15
13
0
40
80
LEUCINE
PHENYLALANINE
ISOLEUCINE
9
7
5
3
VALINE
ASPARTIC ACID
ARGININE
ALANINE
LYSINE
6
4
2
SERINE
TYROSINE
GLYCINE
THREONINE
METHIONINE

Fractionation of dispersed gluten from aluminum lactate solution

This experiment was conducted for the purpose of obtaining comparative results with the fractions prepared from gluten dispersed in aluminum lactate.

As recorded in detail in the section on methods, the fractionation was carried out by varying the pH from 3.1 to 4.8 and four fractions were obtained. The amino acid distribution of the fractions is presented in Table VI. It had been expected that fractions A and B would correspond to the less soluble fractions in Tables III and IV. However, results indicate that they are quite different. In fact, none of the fractions obtained in this experiment exhibited a close similarity in amino acid distribution to any of the fractions listed in Table III or Table IV. There was far less variation in the composition of these four fractions and none of them can be classed as "more soluble" or "more insoluble" in the sense that these terms have been used with the fractions obtained from dispersion in sodium salicylate. It is true that the results for glycine show the same trend as in the other tables but the result for arginine show exactly the opposite trend.

The determining factor in the separation of these fractions is probably the difference in the isolectric points of the fractions. The gradual increase in the content of basic amino acids from Fraction A to D strongly suggests

TABLE VI

The amino acid composition of the fractions obtained from aluminum lactate solution by varying the pH

Fractions	A	B	C	D	
Lysine	1.1	1.2	1.1	1.9	
Histidine	1.7	2.1	2.4	2.9	
Ammonia	5.3	4.9	5.0	4.3	
Arginine	2.8	3.1	3.2	4.2	
Aspartic acid	2.1	2.7	3.2	3.6	
Threonine	2.6	2.6	2.5	2.9	
Serine	4.6	5.0	5.0	5.0	
Glutamic acid	43.5	44.6	45.0	34.0	
Proline	14.7	15.6	14.8	15.1	
Glycine	5.7	4.3	3.0	2.7	
Alanine	2.6	2.5	2.6	2.9	
Cystine	2.1	2.1	2.4	7.6	
Valine	3.4	4.2	4.5	5.2	
Methionine	0.9	1.4	1.6	2.1	
Isoleucine	3.2	3.8	4.2	4.7	
Leucine	6.5	7.3	8.0	8.3	
Tyrosine	5.1	4.5	4.1	3.6	
Phenylalanine	5.4	5.8	6.0	6.6	
N cal/N deter,%	95.5	99.5	99.5	-	
% of total proteins	11.7	33.1	14.1	31.6	Recovery 90.5

All values reported as grams amino acid/17.5 grams nitrogen.

that the isolectric point of the successive fractions may be determined by the relative proportions of these acids, since most of the aspartic acid and glutamic acid is present in the form of amides. The higher the proportion of basic amino acids in the protein, the higher the isoelectric point.

Effect of proteolytic enzymes on gluten and its fractions

During investigations of enzymatic hydrolysis of dispersed gluten in this laboratory (78), it was noted that a precipitate gradually formed in the digestion vessels. The amino acid distribution of the precipitate was quite different from that of whole gluten. It seemed possible that enzymic digestion of the gluten fractions might yield additional evidence concerning the relationship of the fractions. It was therefore decided to carry out digestion of whole gluten and of the fractions prepared from the different types of dispersions.

Enzymatic hydrolysis of dispersed gluten . Before carrying out any investigations on the effects of enzymes on gluten fractions, it was necessary to determine the rate and extent of the action of the enzymes to be used on whole gluten. Four dispersions were prepared in the usual manner in the dispersing agents suitable for the action of the enzymes. Two dispersions in aluminum lactate were digested with pepsin at the ratio of 1:100 and 1:50 by weight of the protein in the dispersion. One dispersion in 10% sodium salicylate with added bisulfite was digested with papain at the ratio of 1:100 by weight of the protein in the dispersion. The other dispersion in 10% sodium salicylate without added

bisulfite was digested with trypsin at the ratio of 1:100 by weight of protein. The rate and extent of the enzymatic hydrolysis were determined by the increases in the amounts of non-protein nitrogen, terminal amino nitrogen, and free amino acid nitrogen in the digests, which had been subjected to precipitation with 10% trichloroacetic acid. This treatment removed undigested protein and probably considerable quantities of large digestion products. The results are given in Table VII. All digestions proceeded regularly and appeared to be largely complete at 48 hours. Results presented later show that only slight increases in free amino acids occurred between 48 and 72 hours (Table VIII).

About 75% of the protein was digested by pepsin or papain after 48 hours, whereas only 35% was digested by trypsin. The rate and extent of digestion is shown in the curves presented in Figures 5 and 6, in which the percentages of non-protein nitrogen and terminal amino nitrogen are plotted against the time of digestion. As shown in Figure 5, digestion with trypsin proceeded slowly, and was nearing completion after 12 hours. Papain and pepsin hydrolyzed the gluten more extensively. The rate of production of material soluble in 10% trichloroacetic acid increased rapidly during the early stages of digestion with papain, and almost ceased after 24 hours. The rate of digestion with pepsin increased rather uniformly, and the digestion continued over 48 hours. Doubling the amount of pepsin accelerated the rate but not proportionately.

TABLE VII

Digestion of dispersed gluten with pepsin, papain, and trypsin, Temperature 37.5°C. All results expressed as mg/ml.

Enzyme concentration	Time of digestion (hour)	0	2	4	6	8	12	24	48
Pepsin: 21.0 µg/ml	Terminal amino N	0.01	0.06	0.09	0.10	0.12	0.14	0.18	0.23
	NPN	0.14	0.53	0.69	0.81	0.93	1.05	1.34	1.64
	Free amino acid N	not detected					0.05	0.06	0.10
Pepsin: 42.0 µg/ml	Terminal amino N	0.01	0.08	0.10	0.12	0.14	0.16	0.23	0.29
	NPN	0.14	0.67	0.86	0.98	1.09	1.23	1.56	1.86
	Free amino acid N	not detected					0.05	0.07	0.11
Papain: 20.0 µg/ml	Terminal amino N	0.01	0.06	0.09	0.11	0.13	0.15	0.19	0.26
	NPN	0.10	0.85	0.99	1.10	1.17	1.28	1.40	1.45
	Free amino acid N	not detected						0.01	0.02
Trypsin: 20.0 µg/ml	Terminal amino N	0.01	0.03	0.05	0.07	0.08	0.10	0.12	0.14
	NPN	0.09	0.25	0.36	0.46	0.55	0.64	0.70	0.74
	Free amino acid N	not detected							0.005

Protein concentration: in pepsin digests 2.1 mg/ml
in papain and trypsin digests 2.0 mg/ml

pH values at start of digestion: for pepsin 3.1
for papain & trypsin 7.0

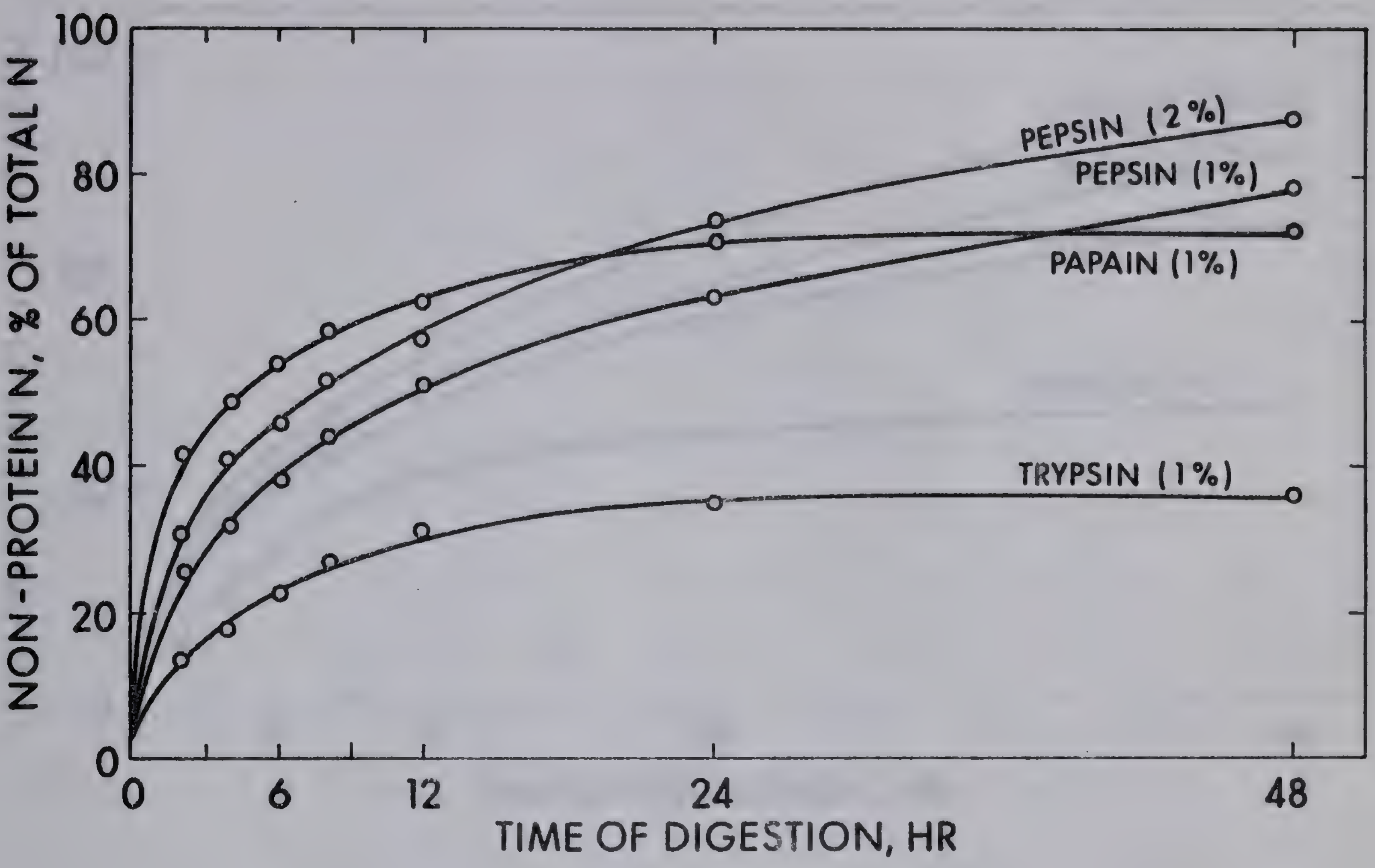


FIGURE 5. Non-protein nitrogen increase in gluten dispersion during enzymatic hydrolysis.

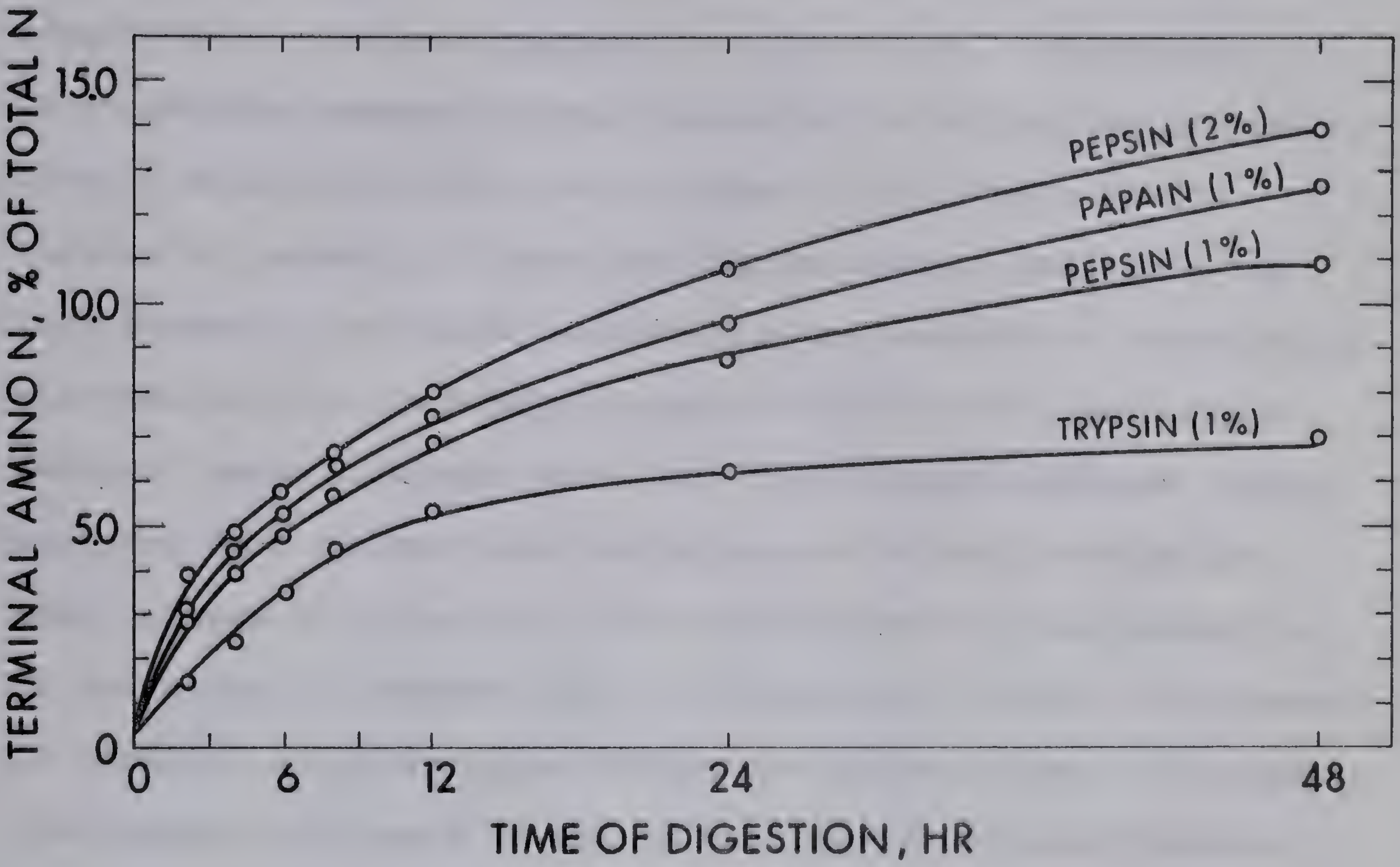


FIGURE 6. Terminal amino nitrogen appearing during enzymatic hydrolysis.

Figure 6 shows the amount of terminal amino nitrogen produced by each of the enzymes. Papain or pepsin produced larger amounts than did trypsin. Papain was slightly more effective at the same apparent concentration, but this is an inadequate measure of the concentration of active enzyme. Considerable difference can be noted in the forms of the curves for papain in these two figures during the early and late stages. The rapid increase in the amounts of non-protein nitrogen during the early stages, but not in the amounts of terminal amino nitrogen indicates that papain produced larger peptides from gluten than did pepsin or trypsin during the early stages of digestion. The falling off in the production of non-protein nitrogen after 24 hours, but not in the release of terminal amino nitrogen during the later stages indicates that papain continued to act on the larger peptides produced at the earlier stages. In contrast, the production of non-protein nitrogen and the release of terminal amino nitrogen by pepsin and trypsin appeared to proceed at essentially similar rates.

After 8 and 24 hours of digestion with papain and pepsin respectively, a precipitate gradually formed, but no such precipitate formed with trypsin. The amounts of proteins precipitated during the digestion of the dispersions by papain and pepsin were about 8.5% and 1.5% respectively of the total protein.

The amounts of free amino acids released during digestion are also given in Table VII. It is shown that the amounts of

free amino acids liberated by papain and trypsin were very small, being less than 8% of the terminal amino nitrogen, but the amounts released by pepsin were considerably higher. About 40% of the terminal amino nitrogen was liberated as free amino acids by pepsin after 48 hours of digestion. It was therefore of interest to determine which amino acids were released from gluten during digestion with pepsin.

The experiments were carried out on Fraction D. The results are given in Table VIII. It is shown that large amounts of threonine, serine, glutamic acid, alanine, valine, methionine, leucine, tyrosine, and phenylalanine, were released. The amounts of leucine and phenylalanine were particularly high, being about 28% of total leucine, and 16% of total phenylalanine in Fraction D. The specificity of pepsin in splitting the peptide bonds of hydrophobic amino acids has been reported (89), hence the release of valine, leucine, tyrosine, and phenylalanine, was expected. The release of hydrophilic amino acids, such as threonine, serine, and glutamic acid, could have been due to the action of naturally occurring enzymes of gluten.

To check this possibility, an autodigestion of Fraction D and a pepsin digestion of heat-treated Fraction were carried out. The heat-treated fraction was prepared by heating Fraction D for 5 minutes in a water bath at 100°C. This treatment has been used by various workers to inactivate the proteolytic enzymes of gliadin (21).

TABLE VIII

Free amino acids liberated from Fraction D by the action of pepsin

Protein concentration: 2.13 mg N/ml

Pepsin added : 0.24 mg/ml

pH 3.1 Temperature 37.5°C

Conditions	With added pepsin				No pepsin added	Heat-treated with added pepsin
Time of digestion(hr.)	0	24	48	72	72	72
Aspartic acid	0.004	0.01	0.06	0.07	0.07	0.05
Threonine+ Serine	0.006	0.25	0.39	-	0.09	0.11
Glutamic acid	0.004	0.23	0.29	0.28	0.09	0.14
Proline	0	0	0.004	0.05	0.003	0.02
Glycine	0.006	0.05	0.07	0.09	0.06	0.03
Alanine	0.004	0.22	0.32	0.39	0.12	0.08
Cystine	0	0	0	0	0	0
Valine	0.006	0.20	0.36	0.37	0.09	0.19
Methionine	0.002	0.33	0.48	0.46	0.06	0.25
Isoleucine	0.004	0.15	0.20	0.18	0.08	0.11
Leucine	0.05	1.47	1.81	2.18	0.38	1.28
Tyrosine	0.002	0.17	0.24	0.25	0.05	0.14
Phenyl-alanine	0.032	0.58	0.79	0.80	0.23	0.54

All values reported as μmoles/ml of digestion.

The autodigestion was performed as for the regular digests but without added pepsin. The amounts of free amino acids released during the digestion are included in Table VIII. It is evident that a considerable degree of enzyme activity was naturally present in the gluten. The amounts of hydrophilic and hydrophobic amino acids released were equally significant, and these enzymes did not account for the large amounts of hydrophilic amino acids released by the pepsin digestion.

The digestion of heat-treated Fraction D was performed as for untreated Fraction D with pepsin added at the same rate. Since the naturally occurring enzymes of gluten had been inactivated by heating, the amounts of free amino acids released as shown in Table VIII represent the action of pepsin only. It is obvious that the amounts of hydrophobic amino acids, especially leucine and phenylalanine, were still high. There were also significant amounts of hydrophilic amino acids released although these amounts were considerably less than for the untreated Fraction D, and the proportions considerably less than for the hydrophobic amino acids.

Theoretically, the results from these two experiments should be additive to give the results obtained with the digest of untreated Fraction D. While this result is obtained for some amino acids such as glycine, isoleucine, and phenylalanine, it is not obtained for others such as valine, methionine, and leucine. The differences are perhaps due to partial denaturation of the protein molecules during heating.

Enzymatic hydrolysis of gluten fractions . Having confirmed that papain and pepsin digestion of gluten resulted in the formation of precipitates in the digestion mixture, the fractions obtained as in the preceding experiments were digested with papain or pepsin using the same procedures as with whole gluten. The digestions were carried out for 24 hours in all cases, and digestion was stopped by freezing after the removal of the precipitates. These digests were used in the study of peptides reported later in this section.

It was observed that the nature and amounts of the precipitates from different fractions were somewhat different. More precipitate was obtained from papain digests than from peptic digests as was expected from the results with whole gluten, and the precipitates obtained from papain digests seemed to be light and fluffy, whereas those from peptic digests were heavier and solid. Precipitates occurred in all fractions prepared from dispersions in sodium salicylate to which bisulfite had not been added, whereas with added bisulfite the precipitates occurred in only the first two fractions.

The precipitates were dissolved in 0.1 N sodium hydroxide solution. All precipitates except those from the digests of Fractions IIS to VIIS dissolved readily, and the solutions were colorless except those from the precipitates obtained with pepsin, which were yellowish. The precipitates from the sulfite-treated gluten were insoluble and no further work was done on them.

Aliquots of the solutions were hydrolyzed with 6 N hydrochloric acid in the usual manner, and the hydrolyzates were analyzed for amino acids. The results are presented in Tables IX and X.

It is clear from the results in Table IX that most of the precipitate that forms when whole gluten is subjected to papain digestion comes from the more insoluble fractions, since about 92% of the total precipitate was produced in the digests of Fractions I to III inclusive. There was a decreasing proportion of the nitrogen in each fraction precipitated as the solubility of the fractions increased with the exception of Fraction VII which is considered not to be from the gluten. The precipitates from all fractions contained much lower proportions of glutamic acid, proline, and cystine, and much higher proportions of lysine, arginine, aspartic acid, and alanine, than did the proteins in the original fractions.

As mentioned earlier, only with the first two fractions were precipitates produced in the digests of Fractions IIB to VIIB, but the total amount of precipitate was approximately equal to the total from the digests of Fractions I to VII (Table IX and X). This appears to confirm the suggestion that the presence of bisulfite in the dispersion results in more sharply defined separation of fractions than was obtained from dispersions with no added bisulfite, and also indicates that gluten can perhaps be separated into two different groups which can be related to the classical glutenin and gliadin; "the more insoluble proteins" which produce the precipitate

TABLE IX

The amino acid composition of the precipitates from Fractions I to VII

Fractions	I	II	III	IV	V	VI	VII	
Lysine	5.9	3.7	3.6	2.8	2.7	2.9	4.3	
Histidine	3.3	3.2	3.4	3.2	3.1	3.0	2.9	
Ammonia	2.7	3.7	4.2	4.2	4.6	3.7	1.7	
Arginine	7.4	6.2	6.3	5.7	4.9	5.6	8.6	
Aspartic acid	9.7	6.7	6.7	5.5	4.6	6.0	7.8	
Threonine	4.2	3.6	3.1	3.1	2.6	3.2	4.9	
Serine	5.8	5.6	5.3	5.3	4.7	5.3	5.7	
Glutamic acid	20.6	29.2	29.6	32.3	35.1	33.1	21.7	
Proline	7.0	9.6	9.8	10.5	11.0	10.5	8.1	
Glycine	5.3	4.9	4.1	3.9	3.4	3.9	6.0	
Alanine	6.2	4.6	4.5	3.7	3.2	3.7	5.0	
Cystine	0.6	0.5	0.2	0.4	0.4	1.9	2.7	
Valine	6.9	6.8	6.3	4.8	4.1	4.6	6.0	
Methionine	2.3	1.9	3.3	1.5	1.2	1.6	1.5	
Isoleucine	5.0	4.9	4.6	4.3	3.8	4.0	3.7	
Leucine	10.2	9.3	8.2	9.3	8.0	8.7	9.4	
Tyrosine	5.0	4.9	3.9	4.5	3.9	4.7	5.6	
Phenylalanine	6.1	5.6	4.9	6.7	6.6	6.5	4.8	
% of ppt in fraction	21.2	18.2	6.1	2.4	1.1	0.9	5.4	
% of ppt in gluten	0.9	5.9	1.2	0.4	0.1	0.1	0.1	Total 8.7

All values reported as grams amino acid/17.5 grams nitrogen.

TABLE X

The amino acid composition of the precipitates from Fractions IIB, IIIB, A, B, and C.

Fractions	IIB	IIIB	A	B	C
Lysine	5.5	3.1	2.8	3.3	2.5
Histidine	3.4	3.2	2.1	2.7	2.5
Ammonia	2.9	3.7	4.5	4.0	3.9
Arginine	7.8	6.1	4.6	4.9	4.4
Aspartic acid	8.3	6.2	4.8	6.6	7.4
Threonine	3.8	3.3	3.1	3.5	3.1
Serine	5.7	5.8	5.0	5.4	5.3
Glutamic acid	21.0	31.0	31.1	31.1	33.4
Proline	8.1	10.8	10.6	10.6	11.2
Glycine	5.1	4.1	5.7	5.3	4.3
Alanine	5.9	4.2	3.9	4.3	3.8
Cystine	0.4	0.6	1.2	1.7	1.5
Valine	6.7	5.9	4.9	5.9	5.8
Methionine	2.5	2.1	0.9	1.8	0.9
Isoleucine	5.0	5.1	3.9	4.8	4.6
Leucine	9.6	9.6	7.1	8.4	8.0
Tyrosine	5.2	4.9	5.0	4.9	4.1
Phenylalanine	6.5	5.7	5.4	6.0	6.0
% of ppt in fraction	44.5	10.9	1.8	3.0	1.9
% of ppt in gluten	5.1	3.5	0.2	1.0	0.3
Total ppt in gluten, %	8.6		1.5		

All values reported as grams amino acid/17.5 grams nitrogen.

after digestion, and "the more soluble proteins" which do not produce the precipitate after digestion. The amino acid content of the precipitates from the digests of Fractions IIB and IIIB as shown in Table X are in excellent agreement with the results of the analysis of the precipitates obtained from the digests of the corresponding fractions from the dispersion without added bisulfite. The results do no account for the much higher (or lower) values obtained for individual amino acids of the precipitates from Fractions IV to VI (Table IX), but the total quantity of these precipitates was relatively small.

The precipitates from the digests of Fractions A to C, as also mentioned earlier, differed from those obtained from other fractions. No precipitate was formed in the digest of Fraction D unless the protein concentration of this fraction was very high. While the total precipitate was much less than with papain digests, there was no essential difference in the proportion of each fraction precipitated during digestion. This behavior was clearly different than with the papain digests. The amino acid content of the precipitates as shown in Table X are similar to those obtained with precipitates from the digests of the other fractions only in that they contained lower proportions of glutamic acid and proline, and higher proportions of the other amino acids than did the protein in the original fractions.

It is interesting to note that an extra peak occurred on the charts of amino acid analysis for the hydrolyzates

of the precipitates from the digests of Fractions A to C. This peak appeared just before the peak for ammonia at the same position as ethanolamine occurred. Assuming that the peak was ethanolamine, the amounts were 0.63 g, 0.59 g, and 0.57 g per 17.5 g N respectively for Fractions A to C. No attempt was made to study this peak.

There were, however, other striking consistencies in the analytical results for the precipitates. In order to illustrate these clearly, results for four amino acids, as obtained from analyses of the total protein in the fractions and of the corresponding precipitates have been brought together in Table XI. For those amino acids that increased substantially with increasing solubility of the fractions there were corresponding increases or decreases in the precipitates of corresponding fractions and, considering the very small quantities involved in the precipitates from the more soluble fractions, the agreement was remarkably regular. This regularity is also apparent in the results for Fraction VII. The results for Fractions IIB and IIIB agree generally very closely with those for the corresponding Fractions I to III. On the other hand, although all precipitates from Fractions A, B and C showed a similar relationship for any given amino acid in the total protein of the fraction and in its corresponding precipitate, there were no significant differences among the three fractions.

These results strongly support the idea that fractionation from sodium salicylate dispersions separates fractions

TABLE XI

Amino acid content of total protein in fractions and of the corresponding precipitates.

Fraction	Glutamic acid		Proline		Arginine		Alanine	
	Total	ppt	Total	ppt	Total	ppt	Total	ppt
I	39.3	20.6	12.4	7.0	4.1	7.4	3.1	6.2
II	41.3	29.2	14.5	9.6	3.6	6.2	2.7	4.6
III	45.2	29.6	15.2	9.8	2.9	6.3	2.1	4.5
IV	46.2	32.3	16.5	10.5	2.4	5.7	2.0	3.7
V	46.5	35.1	17.4	11.0	2.3	4.9	1.9	3.2
VI	34.2	33.1	17.8	10.5	2.5	5.6	2.1	3.7
VII	26.6	21.7	10.5	8.1	6.0	8.6	4.2	5.0
IIB	33.9	21.0	12.0	8.1	4.5	7.8	3.7	5.9
IIIB	42.8	30.9	15.0	10.8	3.1	6.1	2.4	4.2
A	43.4	31.1	14.7	10.6	2.8	4.6	2.6	3.9
B	44.6	31.1	15.6	10.6	3.1	4.9	2.5	4.3
C	45.0	33.4	14.8	11.2	3.2	4.4	2.6	3.8

All values reported as grams amino acid/17.5 grams nitrogen.

with progressively different compositions which can be related to classical gliadin and glutenin, and this separation is sharpened by the use of bisulfite. On the other hand, fractionation from aluminum lactate does not produce such clear-cut separation and does not yield fractions which can be associated with classical gliadin and glutenin.

Since the precipitates obtained on peptic digest and papain digest of gluten are so different in physical properties, it was considered that they might differ in constitution other than amino acid distribution. In the early literature, there was reported a study on the isolation of nucleic acid from the precipitate produced during the peptic digest of wheat germ proteins (1). Therefore, an effort was made to determine nucleic acid in these precipitates. The results showed that a considerable amount of ribonucleic acid was present in the precipitates obtained from the peptic digests of Fractions A, B, and C as given in Table XII, whereas no nucleic acid was detected in the precipitates from any of the papain digests.

TABLE XII

The nucleic acid content of the precipitates obtained from peptic digests of Fractions A, B, and C.
Values reported as mg per 17.5 g nitrogen.

Fractions	A	B	C
Adenosine phosphate	63	32	31
Cytidine phosphate	46	27	26
Guanosine phosphate	32	17	17
Uridine phosphate	58	29	26
Total	199	105	100

Protein associated with nucleic acid in acidic media is well known in the behavior of polyelectrolytes. It is possible that the original proteins in the dispersion may also form such association, but this cannot be established from the present study.

Polypeptide analysis of enzymic digests of gluten fractions. It was considered that examination of the peptides in enzymic digests might give some information about the structural features which distinguish these fractions from one another. In fact, such study was possible only after the fractionation experiments were complete and time permitted only a limited study. The study of the peptides was possible using the amino acid analyzer since characteristic patterns for peptides can be obtained by ion-exchange chromatography.

The chromatography was carried out using two different resins: Aminex 50W-X2 with lower cross-linkages to separate the peptides, and Beckman type AA-15 used for the separation of amino acids and smaller peptides.

The reproducibility of chromatographic curves was checked by running two independent digests. The agreement was generally good, at least qualitatively. Representations of the patterns obtained are shown in Figures 7 and 8.

It is evident from Figure 7 that essentially similar patterns were obtained for papain digests of Fractions II to VI as separated using Aminex 50W-X2. Each of the patterns contained at least 11 similar major peaks, numbered on the diagrams as 1, 2, 5, 6, 7, 8, 10, 12, 14, 15, and 17, although

there were some minor differences. The only major difference found in the pattern of the digest of Fraction II was in the middle section, where some additional peaks, 3, 4, 7, and 9, were obtained.

The pattern of the digest of Fraction I was quite different from those of the digests of the other fractions. Only five major peaks, namely, 1, 2, 4, 11, and 12, were obtained. There were no peaks found between peaks 4 and 11 nor after 12. A significant difference is that peak 11 was found only in this pattern.

The pattern of the digest of Fraction IIIB was also distinguishable from the others. Although the number of peaks showed no real differences, there were appreciable differences in the height of some of the peaks and presumably therefore, in the quantities of the peptides involved.

The patterns obtained for peptic digests of Fractions A, B, and C, were essentially similar but the pattern for Fraction D (See Figure 9), was distinctly different. No explanation for these differences can be offered at this time.

A comparison of the patterns obtained from papain and peptic digests makes it evident that there are wide differences in the action of the enzymes, even though direct comparison of individual fractions is not possible. There are, however, characteristic peaks which clearly distinguish the two types of digests, e. g. the relative prominence of peaks 6 and 12.

Figure 8 shows the chromatographic patterns of enzymic digests of the fractions using Beckman type AA-15. Since the

FIGURE 7.

The chromatographic patterns obtained for enzymic digests of gluten fractions using Aminex 50W-X2.

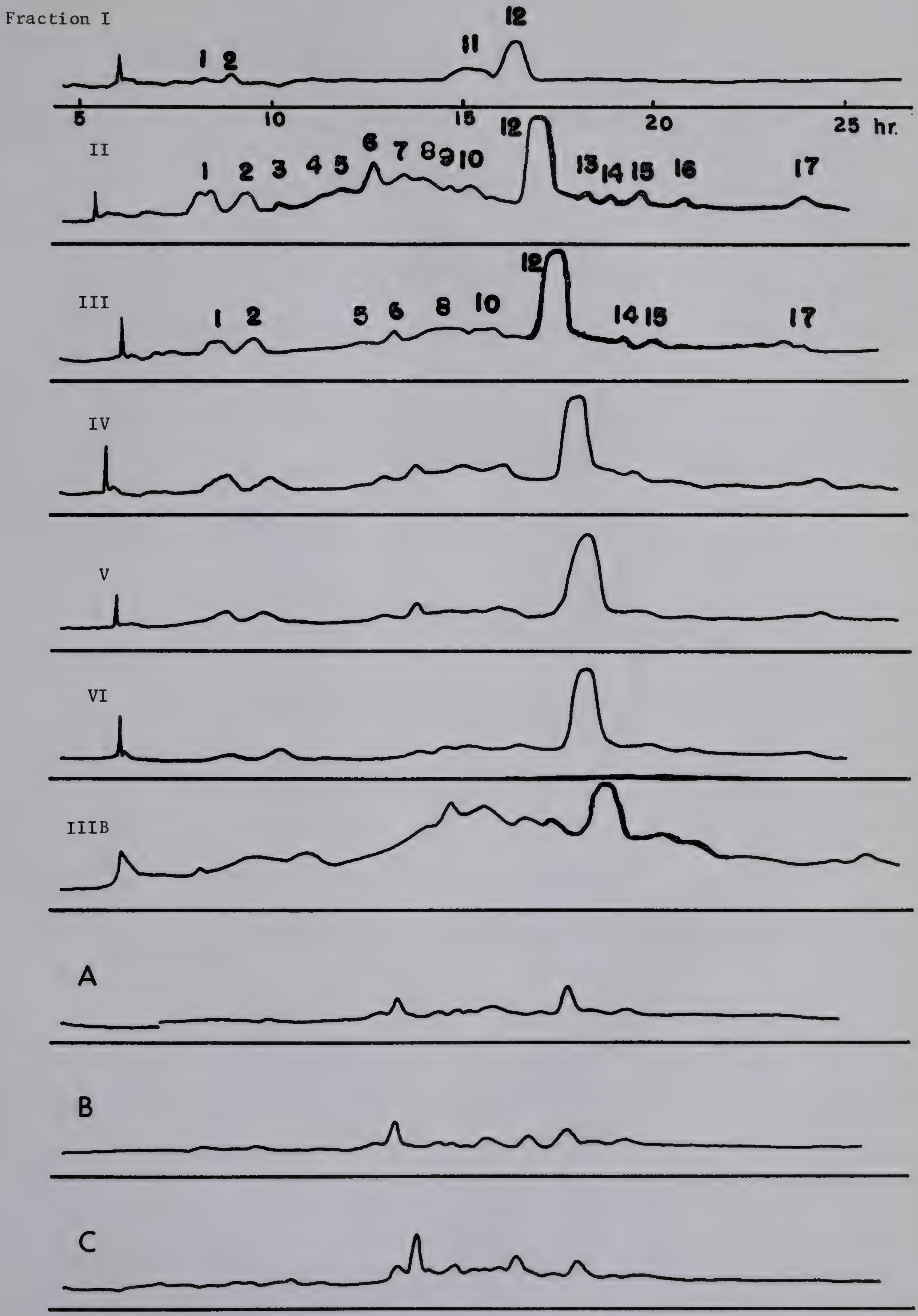
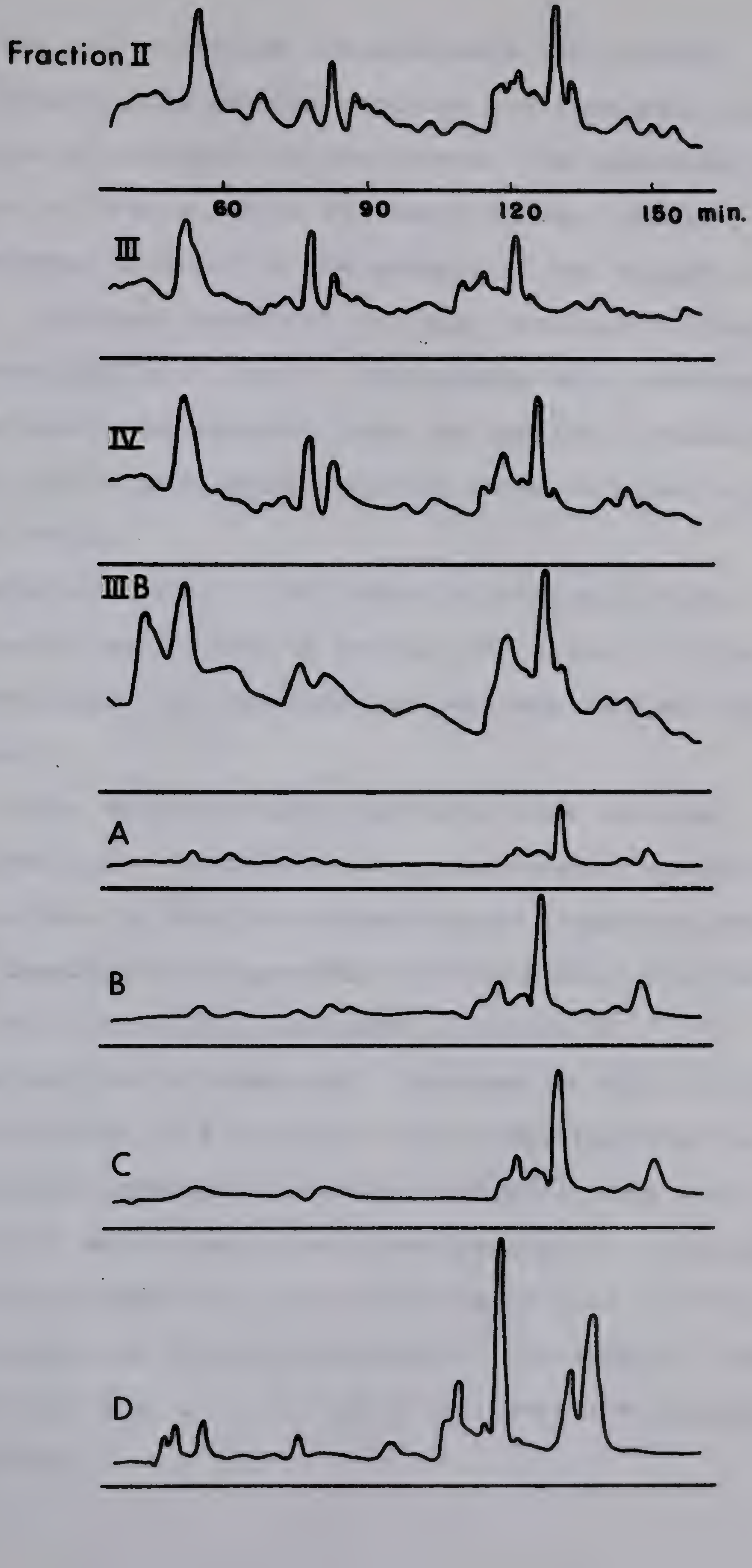
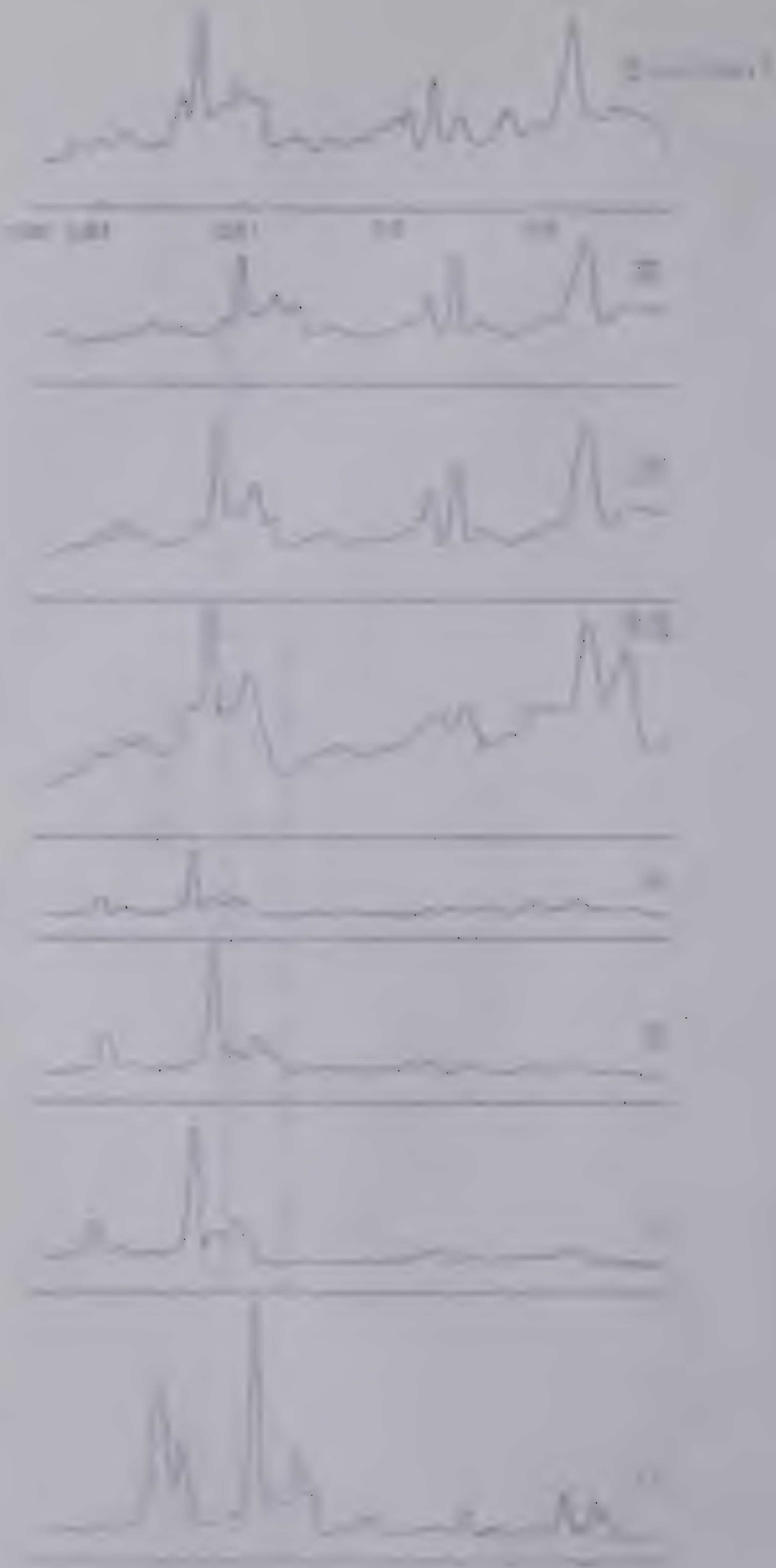

Fraction I
1
2
11
12
5
10
15
20
25 hr.
II
1
2
3
4
5
6
7
8
9
10
12
13
14
15
16
17
III
1
2
5
6
8
10
12
14
15
17
IV
V
VI
IIIB
A
B
C

FIGURE 8.

The chromatographic patterns obtained for enzymic digests of gluten fractions using Beckman type AA-15.


Fraction II
60
90
120
150 min.
III
IV
IIIB
A
B
C
D



larger peptides and undigested proteins were just washed through the column, only smaller peptides and free amino acids would give rise to the peaks in the curves. The patterns for papain digests of Fractions III, IV, and IIIB were similar, but slight differences occurred in the pattern of the digest of Fraction II. Excellent agreement was also obtained for peptic digests of Fractions A, B, and C. More peaks were obtained for Fraction D as was to be expected from the results in Table VIII. These results are in good agreement with those obtained by using the different resin.

It was considered that a full detailed analysis of the peaks of characteristic patterns was not the object of this study. Nevertheless, one complete analysis was carried out using Fraction D.

In fact, these chromatographic patterns were recorded according to the color development of peptides with ninhydrin. An attempt was made to draw the chromatographic pattern according to the color development of peptides with ninhydrin with prior hydrolysis. The results are presented in Figure 9.

It is obvious that a substantial increase in color development after hydrolysis at A indicated that large peptides or perhaps undigested proteins were being eluted at this point. Little difference before and after hydrolysis at B, indicates that most of these peaks are due to free amino acids in the unhydrolyzed fractions giving these peaks. The type of increase in color development at C, D, E, and F indicates the presence of small peptides.

The peaks which showed no color increase after hydrolysis were identified by the use of the amino acid analyzer to be specific amino acids. These results are presented in Table XIII.

TABLE XIII

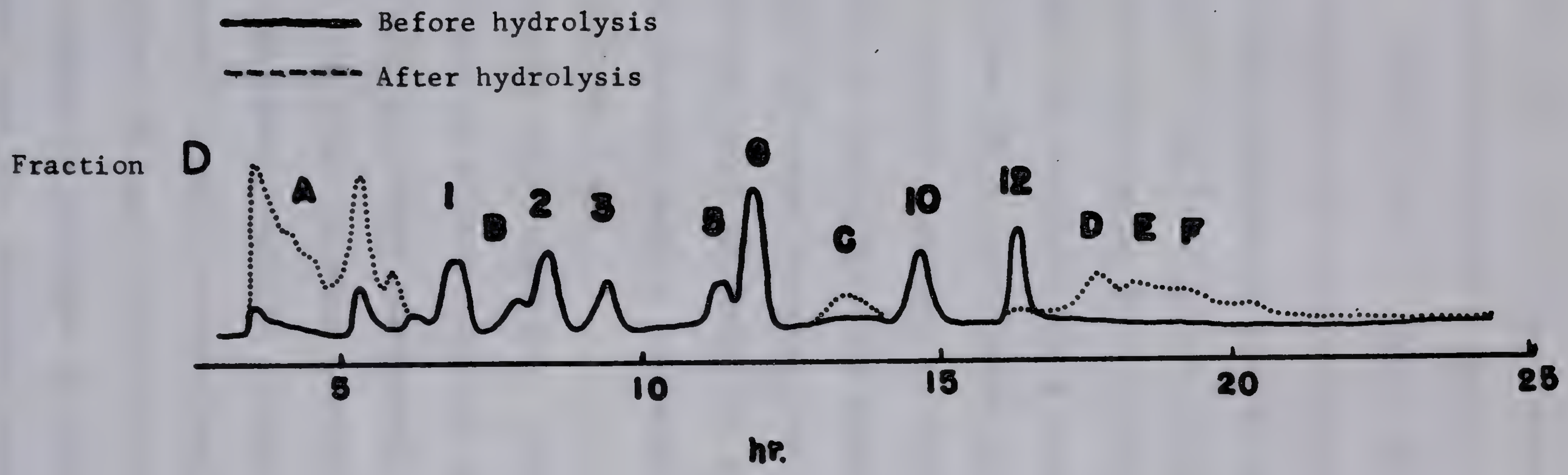
The peaks in Figure 9 identified to be free amino acids

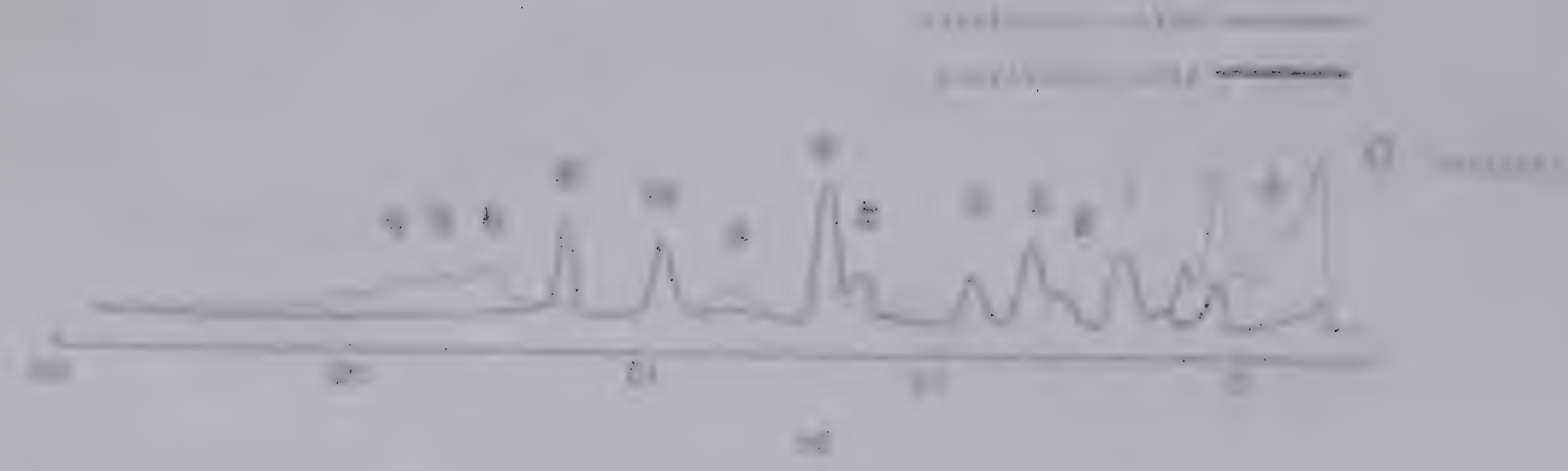
Peak No.	Amino acids
1	Aspartic acid, Serine, Threonine
2	Glycine, Alanine
3	Valine
5	Methionine
6	Leucine, Isoleucine
10	Phenylalanine
12	Ammonia

The peaks which showed only slight to moderate increases in color after hydrolysis were examined by paper chromatography using various solvents (90). The results showed that most of the peaks contained at least 5 different peptides. No further analysis was carried out.

FIGURE 9.

The chromatographic patterns obtained for peptic digest of Fraction D.


Before hydrolysis
After hydrolysis
Fraction D
A
1
B
2
3
5
6
C
10
12
D
E
F
5
10
15
20
25
hr.



DISCUSSION

It has been shown that the cleavage of disulfide bonds by aqueous sulfite solution as shown in the equation,

$$RSSR + SO_3^{--} \rightleftharpoons RS^- + RSSO_3^-,$$

is a highly specific equilibrium reaction. The reaction is at the same time a reduction and an oxidation and provides a specific means of splitting the disulfide bond. Cecil and McPhee (91) have concluded from kinetic studies with simple substances that the reaction is a reversible bimolecular displacement above pH 9, but below pH 9 the mechanism is complex. The rate of reaction is dependent on the net charge in the vicinity of the disulfide bond, a negative charge causing a large decrease in the rate constant. They also concluded that the rate of reaction in the presence of bisulfite ions is negligible compared with that in the presence of sulfite ions.

In the present investigation, it was observed that the addition of sodium sulfite to gluten dispersed in sodium salicylate solution apparently resulted in increased solubility of the less soluble protein to the extent that about 25% of the total protein in the less soluble fractions was not recovered by half saturation of the dispersion with magnesium sulfate (Figure 1 and Table V). There appeared to be little effect of sulfite on the more soluble fractions and this agrees with the results reported by Woychik *et al*.

(60) who found no changes in the number of major components in the gliadin fraction subjected to reduction by mercaptoethanol, although the electrophoretic mobility of these components had been reduced by approximately 20%.

Nielsen *et al*. (59) and Woychik *et al*. (60) suggested that intermolecular bonding is responsible for the very high molecular weight of glutenin. The present results may be interpreted as indicating that intermolecular disulfide bonds are extensively involved in the structure of the more insoluble fractions (glutenin) and that the sulfite treatment ruptured these disulfide bonds. On the other hand, the more soluble fractions (gliadin) were not at all extensively affected by the sulfite treatment and are apparently not dependent on the intermolecular disulfide bond for their basic structure.

Although the reaction which produced the cleavage of disulfide bonds has been termed reversible, it is most unlikely that the reactions in gluten could be reversed and the original properties of the so-called glutenin restored. Nevertheless, a small proportion of the disulfide bonds might be present under the conditions employed in these experiments. This might account for the precipitation of Fraction IIS at a relatively low concentration of magnesium sulfate.

The effect of bisulfite on gluten dispersed in sodium salicylate has been studied by Udy (58). It was claimed that the pronounced effect of bisulfite on viscosity of the dispersion is the result of the cleavage of an appreciable proportion of disulfide bonds in gluten molecules. In the present

study, the results from Figure 1 and Table IV do not indicate this as a major effect.

In fact, it is quite possible that disulfide bonds were not involved in the changes effected by bisulfite. McCalla (92) discussed electrophoretic studies on gluten and concluded that sodium salicylate dispersion eliminates the electrophoretic differences that have been clearly demonstrated for different fractions of gluten. Since the rate of cleavage of disulfide bonds is largely reduced in the presence of negative charges located in the same vicinity, it is probable that the disulfide bonds in gluten dispersed in sodium salicylate are not readily accessible to attack. Consequently, it is suggested that the effect of bisulfite on gluten molecules is through quite a different mechanism, possibly the rupture of the cross-linkages formed by carbonyl compounds as suggested by Hlynka (56). The less drastic modifications may be considered as disaggregation and this could account for the pronounced effect of bisulfite on the viscosity of gluten dispersions, the change on extensibility of dough (93), and the change in the form of the precipitation curves in the present study (Figure 1).

In any general discussion of the results presented in Figures 2 and 3, it is necessary to give some consideration to the possible effects of aggregation of the protein molecules. The dispersing action of sodium salicylate on gluten strongly supports the idea that this reagent causes disaggregation of molecules making up the gluten. Since Naismith (94) has found

considerable evidence for the occurence of aggregation of proteins from the groundnut dispersed in sodium salicylate, it is possible that salicylate may have a similar effect on gluten proteins which would result in the aggregation of molecules of basically quite different amino acid composition. When magnesium sulfate is added to sodium salicylate dispersions, such aggregation would result in preventing clearly defined separation of fractions. Nevertheless, the changes in composition from fraction to fraction is quite regular (Figure 2). The results from the fractionation of dispersed gluten treated with bisulfite (Figure 3), in which aggregation of proteins had been minimized, probably by the rupture of thebonds formed by carbonyl groups, support this idea, since the differences in the composition of successive fractions were increased by the treatment but the general regularity of results was not affected. It is likely therefore that aggregation of the gluten proteins in sodium salicylate occurs to only a very limited extent. Consequently, it seems likely that the differences in distribution of amino acids reflect real differences in the fractions.

The amino acid distribution of gluten components obtained from acid media has been reported by Woychik _et_ _al_. (52) and Beckwith _et_ _al_. (39). The results of their studies are brought together in Table XIV for comparative purposes.

It is possible to consider the components α-2,β, γ, and ω as equivalent to the "more soluble" fractions of the present experiments, since in comparison with α-1, they all contain lower proportions of arginine, aspartic aicd, glycine, lysine,

TABLE XIV

Recent amino acid analyses of gluten components.

	Woychik *et al.*(52)						Beckwith *et al.* (39)	
Gluten components	α-1	α-2	β	γ	ω	water soluble	F-I	F-III
Alanine	2.5	2.3	2.5	2.4	1.5	4.1	2.0	2.3
Arginine	3.1	3.4	2.2	2.0	1.3	6.7	2.6	2.3
Aspartic acid	2.7	2.0	2.7	2.0	1.3	6.8	1.7	3.2
Cystine	1.5	2.0	2.1	2.4	1.4	4.4	2.4	2.2
Glutamic acid	41.1	44.4	46.8	47.5	48.4	22.2	47.7	44.7
Glycine	5.5	4.7	1.6	2.1	2.2	4.3	2.3	1.7
Histidine	1.9	2.8	1.8	1.8	0.8	3.9	2.2	2.2
Isoleucine	3.4	4.7	4.9	4.7	2.4	4.1	4.1	4.5
Leucine	6.8	8.0	8.1	7.0	4.8	7.9	8.3	8.3
Lysine	1.4	0.9	0.8	0.8	0.8	4.5	0.7	0.6
Methionine	1.3	1.8	0.8	1.3	0.9	2.1	1.9	1.3
Phenylalanine	4.8	5.0	5.4	7.2	11.6	4.3	6.4	5.6
Proline	14.8	18.9	20.6	21.1	25.3	11.8	15.2	15.3
Serine	5.6	5.3	4.7	4.2	4.4	5.6	6.4	4.6
Threonine	3.1	2.8	3.0	2.3	2.6	2.8	2.6	2.0
Tryptophan	1.9	1.3	0.7	1.0	0.5	3.1	-	-
Tyrosine	4.5	3.1	3.1	1.9	2.7	4.3	2.2	4.0
Valine	3.9	4.6	5.0	4.0	2.7	6.7	4.6	4.3
Ammonia	5.0	5.7	5.0	5.7	5.0	3.8	5.4	5.4

All figures expressed as grams amino acids per 17.5 grams N.

and tyrosine, and higher proportions of glutamic acid and proline. If this comparison is valid, these components would precipitate from a sodium salicylate dispersion at the concentration of magnesium sulfate in the range within 0.08 to 0.20 saturation, and in the order of the components α-2, β, γ, and ω. The component α-1, so-called glutenin, can also be compared to the "more insoluble" proteins as judged from the values for most amino acids although the content of arginine is quite different. Similarly, the components F-1 (higher molecular weight) and F-III (lower molecular weight) of gliadin obtained by Beckwith et al. (39) can also be classed as the "more soluble" protein and these fractions compare to Fractions IV and V, respectively, in this experiment.

It is clear that separation of "fractions" or "components" using differences in the electrostatic properties of the components to effect separation should be preferable to the use of solubility differences alone. Such properties cannot be used with gluten dispersed in sodium salicylate, but the above discussion confirms that fractions differing in solubility do differ in other properties and that these differences can be related to those of components separated by electrophoretic methods.

The most soluble fraction in gluten, as usually prepared, presumably corresponds to the water soluble proteins of wheat flour, and the amino acid distribution is distinctly different from that of the true gluten. It has been shown by many investigators (eg. 52, 95-97) that the water soluble proteins

contain lower proportions of glutamic acid and proline, and higher proportions of arginine, cystine, lysine, aspartic acid, and leucine. The values for Fraction VII agree in general with the values reported by Woychik et al. (52) for the water soluble proteins (Table XIV), although the latter values show greater differences, as compared with gluten, for most amino acids. It is therefore probable that only part of the water soluble proteins are precipitated in Fraction VII.

Woychik et al. (60) have reported that reduction of the disulfide bonds of wheat gliadin and glutenin followed by electrophoresis in starch gel revealed the presence of some components which are perhaps common to both proteins, and trace components which probably originate from incorporated components comparable to, or identical with, water soluble proteins of wheat. Woychik (98) has further suggested that glutenin may arise through intermolecular disulfide bonding of gliadin components and possibly of water-soluble protein, this suggestion being based on the observation that reduced components of both glutenin and gliadin have some of the same amino acids in the N-terminal positions and that the amino acid composition of the reduced glutenin components is not too dissimilar from those of corresponding gliadin components. Moreover, Ewart (79), on the basis of the results of his study on fingerprinting of performic acid oxidized glutenin and gliadin, has supported this hypothesis.

This argument does not, however, seem acceptable. In fact the gliadin components had threonine and alanine as

N-terminal amino acids as well as aspartic acid, glutamic acid and serine which were also found to occur as N-terminal amino acids in reduced glutenin. Furthermore, Woychik admits that the occurence of electrophoretic components with identical mobilities in reduced gliadin and reduced glutenin "does not in itself confirm the idea that glutenin arises through intermolecular disulfide bonding of gliadin.....". This is particularly true when the results for sulfite-treated gluten in the present experiments are considered. It is certain that the solubility of the reduced glutenin is quite different from that of gliadin. Beckwith et al. (61) have reported that reduced glutenin and gliadin show different solubilities in urea solutions.

Perhaps the most convincing evidence against the idea that glutenin arises primarily through intermolecular disulfide bonding of gliadin components lies in the amino acid distribution in the various components or fractions. Neither the results from the present experiments nor those of Woychik et al. (52) permit this conclusion. The differences in amino acid distribution are far too great to be explained by suggesting that some water soluble protein is incorporated with gliadin to produce glutenin.

Jones et al. (21) have reported that classical glutenin can be obtained from aluminum lactate dispersion when the pH is adjusted to the value of 4.8. The fraction they obtained included appreciable amounts of all fractions of gliadin. The gliadin in this fraction was extracted from the precipitate

with aluminum lactate at pH 3.1, the glutenin remaining insoluble. In the present experiments, fractions were precipitated at pH 4.0, 4.4 and 4.8 (A, B and C) but, on the basis of analytical results, none of these were distinct fractions or "components". It seems clear that there was interaction among the gluten components in aluminum lactate.

Digestion studies of whole gluten indicated clearly that the dispersed gluten was extensively digested by papain or pepsin under these experimental conditions. The results from Figure 5 showed that papain produced larger peptides from gluten than did pepsin during the early stages of digestion, but continued to act on the larger peptides during the later stages. Pepsin, on the other hand, digested the gluten regularly, and appeared to produce only smaller peptides.

Verma and McCalla (78) reported that the sedimentation analyses of the digestion suggested that papain produced large quantities of much smaller products, whereas pepsin produced relatively large quantities of intermediate-sized products. In the present study, the average number of amino acids per peptide can be roughly calculated from Table VII by the ratio of non-protein nitrogen to terminal nitrogen (a correction should be made for free amino acid nitrogen). These calculations show that much smaller peptides were obtained after 48 hours digestion by using papain than by using pepsin. This is in accord with the results obtained by Verma and McCalla.

The amount of free amino acids obtained in the peptic digests of Fraction D was surprisingly high. The results of

Finlayson (76) indicated that only 0.16% of the total glutamic acid, 1.12% of the total serine, 0.15% of the total valine, and 0.80% of the total leucine, in gliadin were released as free amino acids by the action of pepsin and then trypsin. Part of the total was probably produced by the action of the enzymes naturally present in gluten as shown by the results in Table VIII, but these cannot account for the much higher level in the present study. No attempt was made to follow this further. It is possible that the higher pH used during the digestion in this experiment may account for the differences.

Studies of the precipitates obtained from enzymic digests of gluten fractions confirm that the protein producing these precipitates is basically a part of the more insoluble fractions (classical glutenin) of whole gluten, and that the clearer separation of the fractions by the use of bisulfite in the dispersing medium eliminated the precursor of the precipitate from the more soluble fractions (classical gliadin). The amino acid distribution of the precipitates from Fractions I to VII indicated clearly that the proteins producing the precipitates were certainly heterogeneous.

Precipitates were also obtained by Strusi (74) from tryptic digests of gluten from durum and bread wheats, but he presented only qualitative results which cannot be compared with the results of the present study.

The association of RNA in the precipitates obtained from peptic digests of gluten fractions suggested that the adsorption

of RNA on gluten proteins may occur in acid media. However, the possibility that alterations in solubility and electrophoretic behavior of gluten proteins caused by adsorption of RNA would appear to be small, but this cannot be established from the present study.

Studies of the peptides in the enzymic digests of several fractions indicated that more peptides were obtained when the digests were prepared with papain than with pepsin. There was essential similarity in the chromatographic patterns of papain digests of all the more soluble fractions, but there were differences in the patterns for the more insoluble fractions. It seems reasonable to conclude therefore that the more soluble proteins have the more regualr sequential similarity in their structure. The more insoluble proteins, however, appear to have quite different structures as would be expected from the amino acid results. The results of the peptic digest of gluten fractions also indicated that there must be differences in the structure of Fraction D as compared with that of the other fractions. These results are preliminary in nature, but they serve to confirm the differences and similarities found among fractions on the basis of amino acid analyses. Many of the peaks in the chromatographic patterns contained more than a single peptide as indicated by subsequent paper chromatography. A great deal more work would be necessary to obtain completely separated peptides and it is questionable whether the results would justify the work required.

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